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Cross-Reactive CD8 T Cell Responses and Heterologous Immunity During Acute Epstein-Barr Virus Infection: a Dissertation

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CROSS-REACTIVE CD8 T CELL RESPONSES AND HETEROLOGOUS IMMUNITY DURING ACUTE EPSTEIN-BARR VIRUS INFECTION

A Dissertation Presented

By

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ABSTRACT

A person is exposed to many pathogens throughout their lifetime, and with the resolution of each infection, there remains a pool of pathogen-specific immune cells that protect that person from re-infection with the same pathogen. However, there is a great deal of evidence to suggest that the pool of pathogen-specific memory cells can also participate in the immune response to future infections with unrelated pathogens. Many believe T cells to be cross-reactive in nature because of their interaction with self antigens during development in the thymus and their interaction with foreign antigens once in the periphery. There are many features of the interaction between a T cell and its ligand that facilitate this cross-reactive nature. Based on solved crystal structures, relatively few contacts are required for a stable interaction, and that interaction is often mediated by the flexible CDR3 loops of the T cell receptor that accommodate ligands of various structure. There is also evidence in the murine and human systems that subsets of virus-specific memory CD8 T cells take on an activated phenotype upon infection with an unrelated virus. In murine models, these memory T cell subsets could kill target cells, secrete several cytokines, and proliferate in response to a cross-reactive stimulation, suggesting that a cross-reactive T cell response could impact the outcome of a viral infection. In fact, upon heterologous infection, mice immune to a previous virus were often protected, having lower titers of the second unrelated virus, their epitope-specific and T cell receptor repertoires were often skewed, and they were more prone to immune-mediated pathologies. All of these observations coincided with the presence of cross-reactive T cell responses. Thus, we define heterologous immunity as changes in viral

replication and the disease pathology associated with that viral infection as a result of the host's history of infection, and this can be mediated, in part, by cross-reactive CD8 T cell responses.

Since many human viral infections are associated with a wide range of disease states, we questioned whether cross-reactive CD8 T cell responses occurred as commonly as they appeared to occur in the murine models and whether they influenced the outcome of such infections. Epstein-Barr virus (EBV) infects over 90% of the U. S. population and has a large genome with the capacity to encode a multitude of T cell epitopes. The first part of this thesis research focuses on the identification of cross-reactive CD8 T cell responses with specificity for known epitopes derived from EBV, a common human virus. We directed our study to HLA-A2-restricted responses because of the common expression of this MHC Class I allele in the U. S. population. This study resulted in the detection of cross-reactive responses with five different specificities that involved either the immunodominant lytic EBV-BMLF1$_{280}$ epitope or the latent EBNA 3A$_{596}$ epitope. Three of the cross-reactive responses had specificity for epitopes derived from another unrelated, but common, human virus, influenza A virus (IV). Each of these cross-reactive responses had the potential to participate in the collective immune response to acute EBV infection.

EBV is also well-suited as a model system to study heterologous immunity in humans, as infection at an early age is frequently asymptomatic, while the same infection during adolescence often results in an immune-mediated syndrome, infectious mononucleosis (IM). Since older individuals have presumably been exposed to more

pathogens in their lifetime and, therefore, would have memory CD8 T cell pools with more extensive specificities, we hypothesized that acute EBV infection activated cross-reactive memory CD8 T cell responses that promoted the development of IM. In order to determine if the cross-reactive responses we identified above contributed to the immune response to acute EBV infection, we first screened the blood of IM patients for cross-reactive T cells with specificity for EBV-BMLF1$_{280}$ and IV-M1$_{58}$. The total number of M1-specific T cells of 5 of 8 patients was increased at presentation with IM, which was suggestive of their specific activation during the EBV infection since a bystander mechanism would have resulted in 8 out of 8 patients having increased numbers of M1-specific T cells. Our hypothesis was further supported by the fact that we clearly detected cross-reactive T cells capable of recognizing both BMLF1 and M1 epitopes in the blood of 2 of the 5 IM patients with an augmented M1-specific T cell frequency. Furthermore, the M1-specific TCR repertoires of those two patients were dramatically skewed, which was an indication of cross-reactive M1-specific T cell expansions and, therefore, participation in the lymphoproliferation characteristic of IM. In addition, T cell lines derived from 3 out of 8 healthy donors with previous exposure to both viruses contained a subset of T cells that responded to both BMLF1 and M1 epitopes, suggesting that these cross-reactive cells are often maintained in memory. These cross-reactive T cells were cytotoxic and produced MIP-1β, IFNγ, and TNFα, functions which could potentially promote the symptoms of IM and, indeed, may have been contributed to the severe case of IM noted in one patient.

The final part of this thesis research focused on defining the structure of the cross-reactive TCR that recognized both BMLF1 and M1 epitopes, which have only 33% sequence similarity. In addition, we examined the cross-reactive TCR repertoire organization of multiple individuals to determine the breath and, therefore, the likelihood that this cross-reactive T cell response will occur. These studies revealed that a wide range of Vα and Vβ families can mediate interaction with both epitopes and that the cross-reactive TCR repertoire was unique to each individual, relying heavily on the T cell clones present in that individual's private BMLF1- and M1-specific repertoires. We also observed an increased frequency of TCRs with longer CDR3 regions within the cross-reactive repertoire, which were often extended by non-bulky amino acid residues that could provide these TCRs with more flexibility in order to accommodate the two different epitope structures.

Given that we detected a cross-reactive T cell response with specificity for two immunodominant epitopes derived from two of the most common human viruses among people that share one of the most common MHC Class I alleles in the U. S. population, we predict that cross-reactive T cells are common components of human immune responses. The variability in the magnitude and specificity of each cross-reactive T cell response is dependent on each individual's unique history of infection and their unique TCR repertoire, and such responses likely represent one of many factors that could explain the individual variability in disease severity associated with EBV and many other human viral infections.

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ABBREVIATIONS

aa: amino acid

APC: antigen-presenting cell

APL: altered peptide ligand

BMLF1: (EBV-derived early lytic protein) BamHI M fragment first leftward ORF

BRLF1: (EBV-derived immediate-early lytic protein) BamHI R fragment first leftward ORF

Cα: constant region of the TCR α-chain

Cβ: constant region of the TCR β-chain

CDR: complementarity-determining region

CMV: human cytomegalovirus

Dβ: diversity region of the TCR β-chain

EBNA: EBV nuclear antigen

EBV: Epstein-Barr virus

HCV: hepatitis C virus

HIV: human immunodeficiency virus

HLA: human leukocyte antigen (human MHC)

IM: infectious mononucleosis

IV: influenza A virus

Jα: junctional region of the TCR α-chain

Jβ: junctional region of the TCR β-chain

LCMV: lymphocytic choriomeningitis virus

LMP: (EBV-derived) latent membrane protein

M1: (IV-derived) matrix protein

MHC: major histocompatibility complex

NP: nucleoprotein

ORF: open reading frame

PBMC: peripheral blood mononuclear cells

pMHC: peptide-MHC complex recognized by a TCR

PV: Pichinde virus

TCR: T cell receptor

Vα: variable region of the TCR α-chain

Vβ: variable region of the TCR β-chain

VV: vaccinia virus

CHAPTER I:

INTRODUCTION

Memory CD8 T cells represent a vital component of the immune system that provide protection against re-infection with previously encountered pathogens, such as viruses. Memory T cells have been primed and are able to interact with peptide-MHC (pMHC) complexes on non-professional antigen-presenting cells (APC) in peripheral tissues. These memory cells are present at higher precursor frequencies than naïve T cells and have fewer requirements for activation, as they are able to respond to lower concentrations of antigen and are less dependent on co-stimulatory signals (reviewed in (Dutton et al., 1998; Sprent and Surh, 2002; Welsh et al., 2004)). Thus, antigen-specific memory CD8 T cells are at the site of infection earlier, are initially present at higher numbers, and are more easily activated than antigen-specific naïve CD8 T cells. These characteristics put memory cells at an advantage when fighting re-infection with an invading virus.

There are currently many examples, in both murine and human systems, which suggest a CD8 T cell can recognize and respond to more than one viral antigen (Anderson et al., 1992; Brehm et al., 2002; Kuwano et al., 1991; Mathew et al., 1998; Mongkolsapaya et al., 2003; Nilges et al., 2003; Wedemeyer et al., 2001). If T cell cross-reactivity occurs frequently, then memory CD8 T cells are not only at an advantage when fighting re-infection with a previously encountered virus but, for the same reasons, a subset of cross-reactive memory cells are also at an advantage when confronted with a new, unrelated, virus. Evidence generated in controlled experiments using a murine

model system of heterologous virus infection suggests that cross-reactive CD8 T cell responses commonly occur and can influence the outcome of infection. Our lab has identified several cross-reactive CD8 T cell responses and has demonstrated that immunity to a previous viral infection can 1) provide at least some protection against challenge with an unrelated virus, as evidenced by lower virus titers and 2) result in immune-mediated pathology following challenge with an unrelated virus, which is distinct from the disease pathology typically observed in naïve mice challenged with the same virus (reviewed in (Welsh and Selin, 2002)). We have termed this phenomenon heterologous immunity.

The goal of this thesis research was to determine if cross-reactive CD8 T cell responses were common during human viral infections and if they influenced the outcome of an infection. To determine whether cross-reactive CD8 T cell responses were a common component of the human immune response to viral infection, we focused our research on one of the most commonly expressed MHC Class I alleles in the U. S., HLA-A*0201, and concentrated our efforts on the immune response to two of the most common human viruses, Epstein-Barr virus (EBV) and influenza A virus (IV). Using these guidelines, we screened the blood of several individuals for the presence of cross-reactive CD8 T cells. T cell receptor (TCR) repertoire analyses were also employed to reveal any structural requirements a TCR must meet to engage more than one ligand, which may help determine how often such cross-reactive TCRs appear within a given memory T cell pool and among different individuals. EBV infection was also a good model system for determining whether heterologous immunity influenced the outcome of

human viral infections. Primary EBV infection later in life frequently results in a CD8 T cell-mediated pathology known as infectious mononucleosis (IM), so we aimed to collect blood samples from IM patients throughout the acute phase of their infection to monitor the presence of cross-reactive CD8 T cell responses and disease severity.

This introduction is separated into more detailed discussions on individual topics as they pertain to this thesis research.

A. Definition of T cell cross-reactivity

T cell cross-reactivity is defined here as the basic biological observation that a given T cell can interact with multiple ligands presented by the same self-MHC molecule. However, it is noted that T cell cross-reactivity also includes T cells that recognize allogeneic MHC molecules (ie. allo-reactivity) as well as T cells that recognize cross-species MHC molecules (ie. xeno-reactivity). This thesis is focused on defining virus-specific cross-reactive CD8 T cell responses and demonstrating the biological consequences of these responses on human disease. In keeping with this focus, virus-specific cross-reactive CD8 T cell lines and clones have already been identified. Viral cross-reactivity can involve the recognition of different proteins expressed by the same virus (Anderson et al., 1992; Kuwano et al., 1991), different viral serotypes (Mathew et al., 1998; Mongkolsapaya et al., 2003), and unrelated viruses (Acierno et al., 2003; Nilges et al., 2003; Wedemeyer et al., 2001). However, it is still unclear how prevalent cross-reactive T cells are among the human population and what role they play in the immune response to viral infection.

During their development in the thymus, CD4 and CD8 single-positive T cells

undergo positive and negative selection processes (reviewed in (Starr et al., 2003)). Positive selection requires a productive, but low avidity, interaction with a self-peptide in the context of a self-MHC molecule which elicits survival and maturation signals. In contrast, a high avidity interaction would reveal a T cell's autoimmune tendency and would signal the negative selection or deletion of that T cell from the naïve repertoire. Thus upon entering the periphery, a naïve T cell already has a low level of specificity for a self-peptide. In order to fully mature into an effector or memory T cell, this naïve T cell must also interact with a foreign peptide. Experiments that demonstrated a naturally processed self-peptide was recognized by a defined antigen-specific T cell clone during its development in the thymus help support the claim that each effector and memory T cell in the periphery is cross-reactive in nature (Hogquist et al., 1997; Hu et al., 1997; Sasada et al., 2001; Tallquist et al., 1996).

The most comprehensible mechanism of cross-reactivity involves molecular mimicry, where a T cell can use the same receptor elements to interact with multiple different ligands that are largely similar in sequence and structure (Figure 1) (Anderson et al., 1988; Fujinami and Oldstone, 1985; Guillet et al., 1987; Welsh and Selin, 2002). However, it is now clear that cross-reactive mechanisms can be more complex, as a T cell can also interact with peptide ligands having little to no sequence or structural similarity (Bhardwaj et al., 1993; Boen et al., 2000; Evavold et al., 1995; Reiser et al., 2003; Zhao et al., 1999). Under such circumstances, the T cell receptor (TCR) may use different receptor elements to interact with each ligand, referred to as an alternative recognition mechanism (Figure 1). The lack of conserved structural requirements for interaction

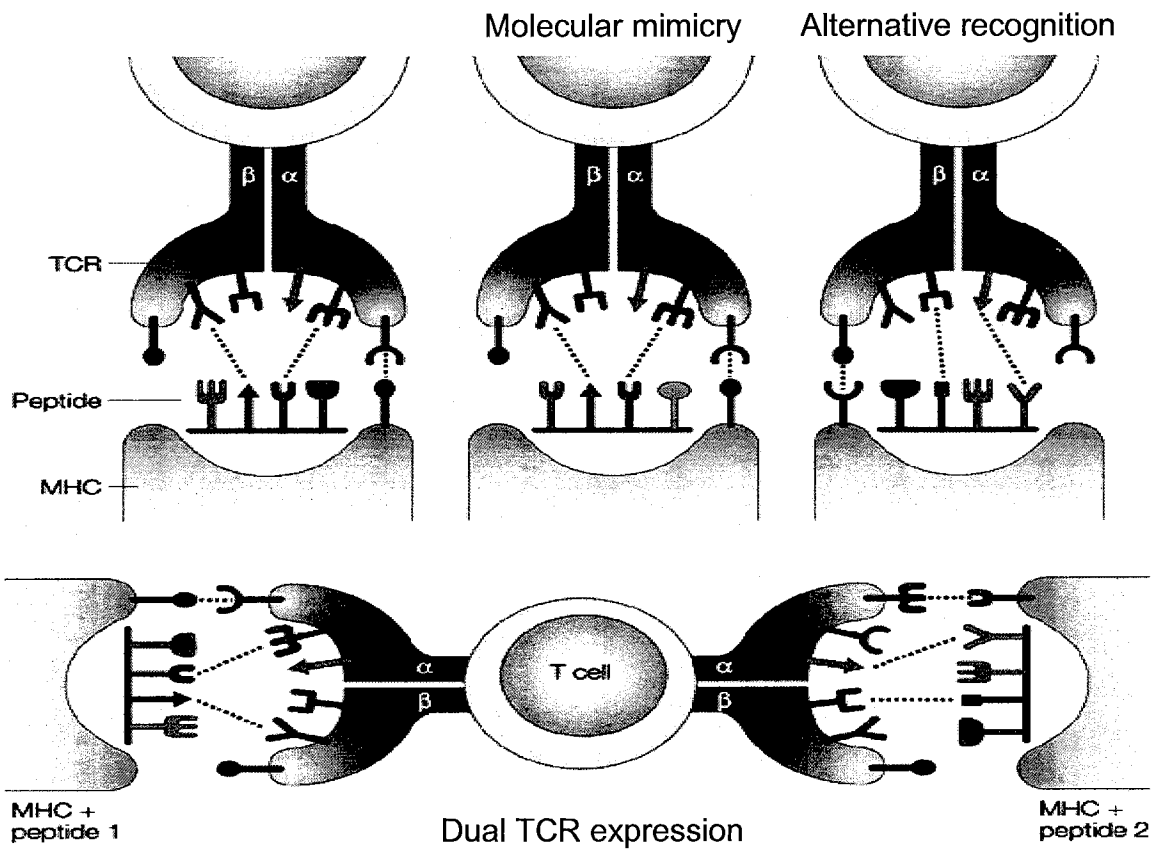


Figure 1. Potential mechanisms of interaction between a cross-reactive TCR and its alternative ligand. This model was taken from Welsh and Selin 2002 Nat Rev Immunol 2:417-426.

between a given TCR and its ligand makes predicting cross-reactivity difficult. Nevertheless, cross-reactive T cell interactions are known to occur in nature and can be an active component of the host immune response (Brehm et al., 2002; Cornberg et al., 2005a; Selin et al., 1994; Urbani et al., 2005; Wedemeyer et al., 2001). This thesis will address how often such cross-reactive T cell responses occur and if they influence the outcome of a human viral infection.

B. Cross-reactive CD8 T cells and heterologous immunity

Heterologous immunity has been described in murine models of viral infection, whereby memory CD4 and CD8 T cells specific to a previous infection protect the host, at least partially, from a subsequent unrelated infection and can also change the disease pathology associated with that infection (Selin et al., 1998; Welsh and Selin, 2002). For example, a memory T cell response to lymphocytic choriomeningitis virus (LCMV) protected mice against infections with either Pichinde virus (PV) or vaccinia virus (VV) based on reduced viral titers and survival to an otherwise lethal dose of VV. The mechanism behind this protection is still under investigation and is likely to depend on the virus involved. While cytotoxicity may play a prominent role in the context of one infection, cytokine production may be more important in the context of another. In the case of a VV challenge, it was determined that IFNγ played a role in the reduction of virus titer (Chen et al., 2001; Selin et al., 1998). LCMV-specific memory CD8 T cells detected at the site of infection correlated with this protection and were shown to produce IFNγ following this cross-reactive VV stimulation (Chen et al., 2001). Furthermore, our lab has more recent evidence that the transfer of cross-reactive LCMV-specific T cell

lines alone could mediate a reduction in VV titer, the extent of which appeared to be influenced by the cross-reactive specificity and TCR repertoire of those T cell lines (Cornberg et al., 2005a). Individual variations in cross-reactive specificity patterns and the cross-reactive TCR repertoire can influence heterologous immunity and are discussed later under the topic of private specificity.

Cytotoxicity and cytokine secretion by cross-reactive memory CD8 T cell populations can also promote or alter the disease pathogenesis associated with a viral infection. While surviving an otherwise lethal dose, LCMV-immune mice often presented profound immunopathology as a result of acute VV infection that differed with the site of infection. An intraperitoneal infection resulted in T cell infiltration of the fat and a condition known as acute fatty necrosis (Selin et al., 1998; Yang et al., 1985). Intranasal infection resulted in the infiltration of LCMV-immune T cells into the bronchus-associated lymphoid tissue and, in some cases, a condition known as bronchiolitis obliterans, where the bronchioles are obstructed with fibrin and inflammatory cells (Chen et al., 2001). This is opposed to pulmonary edema more typical of acute VV infection, where the airways in the lung are fluid-filled. The presence of IFNγ appeared to play a role in both acute fatty necrosis and the regional expansion of bronchus-associated lymphoid tissue and, as stated above, we demonstrated that cross-reactive LCMV-specific memory cells can contribute to the total level of IFNγ production. Activated CD8 T cells can also produce significant levels of TNFα, which has been shown to initiate pulmonary tissue damage during an influenza virus infection (Xu et al., 2004). We are currently investigating the role of TNFα in these VV-

associated pathologies and any contribution made by these activated cross-reactive T cells.

The potential role of cross-reactive T cells in the disease pathogenesis associated with human viral infections is now being appreciated. For example, it is known that cross-reactive CD8 T cells exist that recognize highly similar epitopes derived from different serotypes of Dengue virus and that the activation and proliferation of T cells correlated with heightened disease severity, or the development of Dengue hemorrhagic fever (Mathew et al., 1998; Mongkolsapaya et al., 2003; Zivna et al., 2002). One speculated mechanism for this disease pathology involves TNFα, which has been shown to cause capillary plasma leakage and can lead to shock (Rothman and Ennis, 1999; Vassalli, 1992). Cross-reactive T cell responses may also influence the immune response to hepatitis C virus (HCV) infection, as a subset of IV-specific memory cells have been shown to co-recognize a highly similar epitope derived from HCV, and the proliferation of these cells correlated with severe acute liver pathology and resulted in a chronic HCV infection (Urbani et al., 2005). Thus, an increasing amount of evidence is suggestive that an individual's history of infection largely impacts future immune responses, and this may be one of the many factors that explain the individual variation in the disease state associated with a given viral infection.

C. T cell activation by altered peptide ligands

Given their potential role in virus protection and associated disease pathologies, investigations into the extent of activation and the resulting effector functions of cross-reactive T cells are paramount. Synthetic variants of a native peptide, termed altered

peptide ligands (APL), have been shown to stimulate a given TCR to its fullest extent (agonist), to a lesser extent (partial or weak agonist), or not at all (antagonist) due to their inability to induce a signal and their ability to block interaction with nearby agonists (Sloan-Lancaster and Allen, 1996). While a low avidity interaction with an APL is frequently insufficient to elicit full effector function, an increase in the concentration of that APL, combined with the signal generated from the CD8 co-receptor, has been reported to compensate for such a weak or transient TCR engagement (Couedel et al., 1999; Holler and Kranz, 2003; Reichstetter et al., 1999). These data suggested that a threshold of T cell activation existed, perhaps for each individual function, and that in order for a T cell to reach its functional potential, this threshold(s) must be met. Alternatively, there is evidence to suggest that the TCR signal generated following ligation with an APL is qualitatively different from that following ligation with the native peptide (Madrenas and Germain, 1996; Sloan-Lancaster and Allen, 1996). These data suggested that the functional response of a T cell may be dependent on the structure of the ligand and that there are no compensatory mechanisms with which to gain full effector function upon interaction with a variant ligand. Based on this theory, each peptide ligand may be responsible for inducing an allosteric change that accommodates binding between the antigen presenting cell (APC) and T cell co-receptors and initiates a unique signal transduction cascade (Madrenas and Germain, 1996).

There are still other models that have been proposed to explain the mechanism behind the differential T cell functions elicited by an APL, thus, the true mechanism is still unknown and may involve aspects of several current models (Madrenas and

Germain, 1996). What is clear is that the functions resulting from TCR engagement with an APL can have profound biological effects. In the periphery, mature T cells that engage APLs can become anergic or secrete an altered pattern of cytokines that not only influences its own function but influences the differentiation and function of neighboring immune cells (Windhagen et al., 1995). We predict that cross-reactive interaction with natural APLs will have a similar effect and that this may impact the development of an efficient immune response and promote T cell-mediated pathogenesis.

D. Concept of private specificity

As alluded to earlier, during the discussion of heterologous immunity, individual variability in the cross-reactive specificity patterns and in the cross-reactive TCR repertoire have the potential to influence the outcome of a viral infection. For instance, while LCMV-immune mice are generally protected from a lethal VV challenge, the extent of the reduction in VV titer and the degree of associated immunopathology varied between individual mice, results which were suspected to be an effect of the different cross-reactive specificity patterns that occurred (Kim et al., 2005). In addition to the observed differences in the specificity of the cross-reactive T cell responses of each mouse, cross-reactive T cell lines having similar specificity were derived from some of these mice but were comprised of different cross-reactive T cell clones with variable avidity for their VV-encoded ligand (Cornberg et al., 2005a). Thus, clonal differences may have influenced the level of protection these cross-reactive T cell lines conferred when transferred to VV-challenged hosts.

Individual variations of the cross-reactive specificity pattern and of the cross-

reactive TCR repertoire likely reflect the unique clonotypic composition of each host's T cell repertoire prior to VV challenge. An investigation of naïve T cell repertoires revealed clonal variations between genetically identical mice, presumably a result of the random TCR gene rearrangements and the stochastic selection pressures endured during T cell development (Bousso et al., 1998). These clonal variations can cause variations in the antigen-specific T cell repertoires of individual mice following the same immunization or infection, which may be even more apparent due to the added stochastic process of encountering their specific antigen presented on the appropriate APC (Bousso et al., 1998; Cibotti et al., 1994; Cornberg et al., 2005b; Kim et al., 2005; Lin and Welsh, 1998). Therefore, despite the possibility of having comparable epitope-specific hierarchies, the clonal composition of each of those epitope-specific responses will differ between individuals, and this phenomenon has been referred to as the private specificity of each individual's T cell repertoire.

While the private specificity of each antigen-specific T cell repertoire ensures that each immune response is unique, often there are also public features of each repertoire that are shared between unrelated individuals. T cells activated by a given peptide-MHC complex generally share conserved TCR features that are necessary for a stable interaction, such as conserved Vα or Vβ family usage or the expression of CDR3 amino acid motifs. It has been postulated that the public specificity of a T cell repertoire is influenced by the structure of the peptide ligand; for instance, a peptide lacking prominent side chains may require contact with a strict, and therefore public, TCR structure having the features necessary for contact (Stewart-Jones et al., 2003; Turner et

al., 2005). According to this theory, a peptide with upfacing side chains of its own may be able to mediate contact with a wider variety of less conserved TCR structures (Turner et al., 2003; Turner et al., 2004). While peptide structure may indeed play a role in the formation of an antigen-specific T cell repertoire, it is unlikely to be the only factor involved.

Unlike public features of a repertoire which may be easier to predict, the private specificity of each repertoire adds another layer of complexity to the study of T cell cross-reactivity. The individuality of each T cell response makes comparing patients difficult and may prevent any generalizations from being made regarding the presence and influence of a given cross-reactive CD8 T cell response on an acute viral infection. However, the private specificity of each individual's T cell repertoire is likely to be another factor contributing to the variability in disease severity associated with many human viral infections.

E. T cell receptor structure and recognition of the peptide-MHC complex

Given the potential influence of the T cell repertoire on human disease and the need to determine how frequently a cross-reactive T cell interaction occurs, it is important to understand the basic principles of TCR structure and the interaction between a TCR and its peptide-MHC complex (pMHC). Such knowledge may reveal how easy or difficult it is for a given TCR to engage a variant ligand, particularly in a case where the putative variant ligand has little structural similarity to the native ligand.

We chose to focus our initial research on CD8 T cell responses restricted by HLA-A*0201, one of the most common MHC Class I alleles in the U. S. population and

representing 55% of our study cohort. HLA-A2*0201 preferentially binds peptides 9 amino acids (aa) in length that have a specific binding motif. Positions 2 and 9 of the peptide sequences commonly contain I/L or L/V residues, respectively, and the side chains of these anchor residues generally insert into two pockets at the opposite ends of the peptide-binding groove (Engelhard, 1994).

The TCR that recognizes these pMHC complexes exists as a heterodimer of one alpha (α) and one beta (β) chain in association with a membrane-bound complex of CD3 proteins (Janeway et al., 1999). The α-chain of the heterodimer is comprised of one variable (V), one junctional (J), and one constant (C) gene, while the β-chain is comprised of one V, one diversity (D), one J, and one C gene. The presence of multiple V, D, and J genes enhances the variability of the developing TCR repertoire, and the various combinations of these genes, through the process of V(D)J recombination, further enhance this variability. Upon joining two of these genes, some of the germline encoded nucleotides may be lost and some non-germline encoded nucleotides may be added to the junction region (N-additions). All of these factors contribute to the diversity of the TCR repertoire so that it may recognize a wide range of foreign antigens.

To fully understand the structure and function of cross-reactive T cells as they pertain to human disease, it is important to review the current theories surrounding the interaction between a TCR and the pMHC. Contact with the pMHC is generally mediated by 6 loops that protrude from the TCR heterodimer, 3 from each chain (Figure 2). Therefore, these loops have been termed the complementarity-determining regions (CDR) because they form a surface complementary to the antigen. CDR1 and CDR2 of

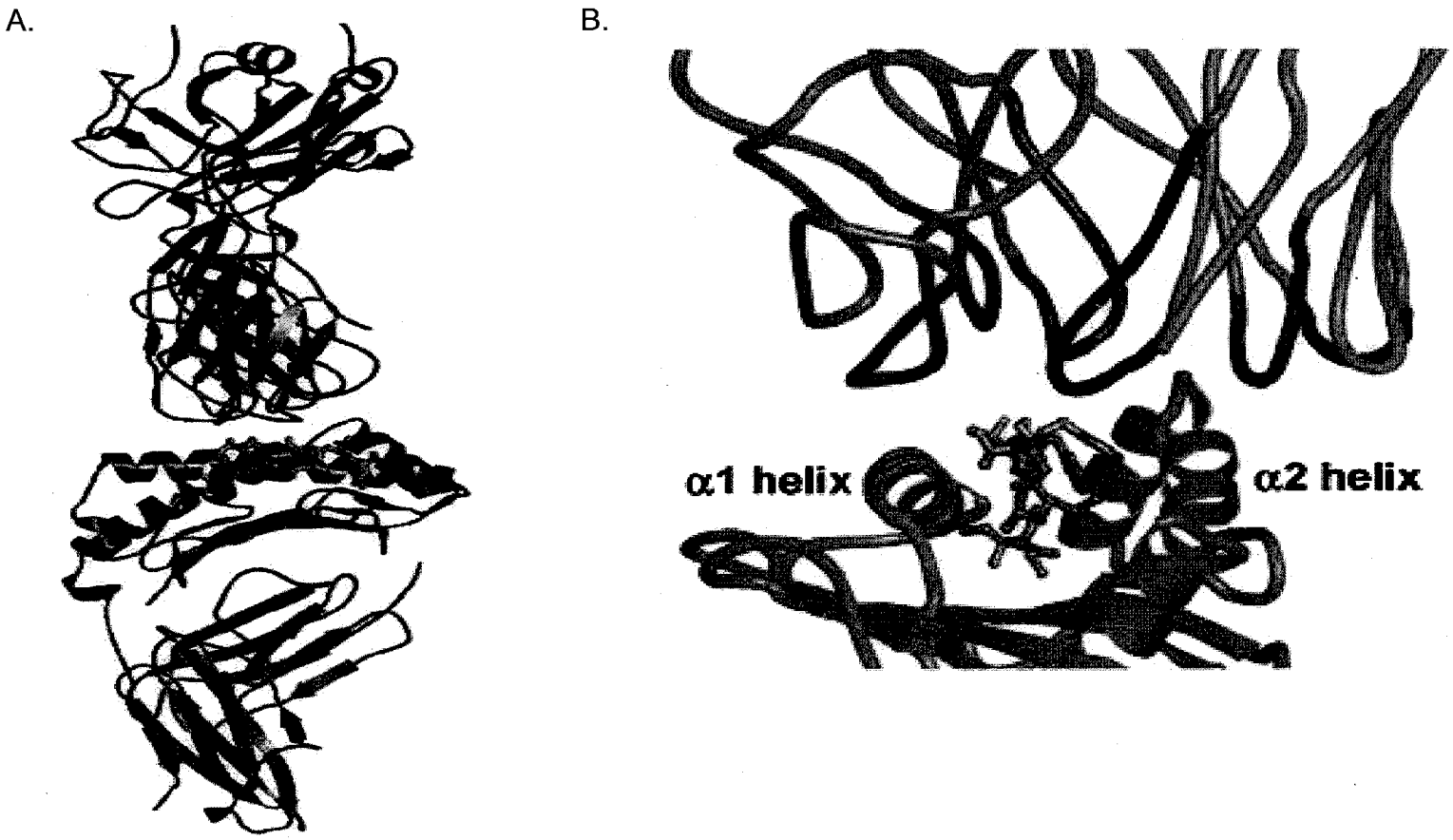


Figure 2. Region of contact between a TCR and its specific peptide-MHC ligand. (A) Diagram representing the components involved in the interaction between a T cell receptor (cyan α-chain, purple β-chain) and its ligand (yellow peptide epitope, green MHC Class I heavy chain, red β_2m). (B) A more focused view of the region of contact during this interaction, where each colored loop represents CDR loops 1-3 for the TCR α- (green, red, blue respectively) or β-chain (purple, orange, cyan respectively) and the peptide epitope is shown in yellow. These diagrams were taken from Stewart-Jones et al. 2003 Nat Immunol 4:657-663.

each chain are germline encoded by the V gene, therefore, T cell clones expressing the same V gene have little diversity between them in these regions. However, CDR3 represents the junction region where the V, D, and J genes combine, making this the most variable sequence of the TCR. The sequence of the CDR3 loop defines each unique T cell clone and, therefore, is commonly used for TCR repertoire analyses.

Based on at least 20 solved crystal structures, 8 of which involve a human TCR bound to peptide presented by HLA-A2, it is thought that the TCR orients itself diagonally over the peptide-MHC complex (Buslepp et al., 2003; Chen et al., 2005; Garcia et al., 1999; Housset and Malissen, 2003; Maynard et al., 2005; Rudolph and Wilson, 2002; Stewart-Jones et al., 2003). Although, there are exceptions to this rule, such as the orthogonal orientation of the *JM22* TCR over influenza $M1_{58-66}$/HLA-A*0201 (Buslepp et al., 2003; Stewart-Jones et al., 2003). Due to this semi-conserved orientation, the TCR α-chain is generally positioned over the amino-terminus of the peptide and the TCR β-chain is generally positioned over the carboxy-terminus of the peptide. Although both chains are involved in the interaction with the pMHC, the solved crystal structures would suggest that slightly more of the interaction is mediated by the α-chain, which can make significant contributions to the stability of the complex (Rudolph and Wilson, 2002). The kinetics of the interaction have been described in a 2-step model whereby the TCR first makes contact with the MHC molecule, which facilitates peptide scanning, followed by a stable interaction between the TCR and its native peptide ligand (Wu et al., 2002). In this model, TCR docking to the MHC, or step 1, is likely mediated through the CDR1 and CDR2 loops of the TCR, which have a more rigid structure and less variable

sequence than the CDR3 loops (Chothia et al., 1988; Davis and Bjorkman, 1988; Garcia et al., 1999; Wu et al., 2002). Contact with the peptide, or step 2, is likely mediated through the CDR3 loops that are better positioned over the central area of the peptide so to contact any side chains that are upfacing (Figure 2). Furthermore, the more flexible nature of the CDR3 loops allows them to fold over the peptide and make more stable contacts (Wu et al., 2002). However, there has been some controversy over the validity of this model as examples arise that do not appear to fit the rule. A revision of the model has since been suggested whereby the peptide ligand involved dictates which CDR loops carry the largest energetic burden of the interaction (Borg et al., 2005). Peptide ligands can vary significantly in their structure, which may bulge out towards the TCR presenting prominent side chains or may lay rather featureless buried within the MHC binding groove. One theory that has been put forth in the field is that peptides with fewer prominent features may require a strict TCR structure with prominent features of its own to initiate contact with the pMHC (Stewart-Jones et al., 2003; Turner et al., 2005). This theory appears to apply to at least one of the peptides used in our research, IV-M1, and it will be interesting to test it in the context of CD8 T cell cross-reactivity (Stewart-Jones et al., 2003).

F. Cross-reactive T cell receptor structure

The knowledge and concepts that have evolved from previous studies of the TCR-pMHC interaction, as briefly reviewed above, have led to several potential mechanisms behind the interaction between a cross-reactive TCR and its alternative ligand (Figure 1). Although it may be an exception to the rule, up to 30% of T cells in the periphery are

thought to express two different TCRs on their surface. Dual TCR expression can result from the expression of two different β-chains or, more commonly, from the expression of two different α-chains due to inefficient allelic exclusion during development (Balomenos et al., 1995; Casanova et al., 1991; Davodeau et al., 1995; Padovan et al., 1993; Padovan et al., 1995). Using a murine system, both TCRs were shown to be functional and, thus, it has been speculated that dual TCR expression is one mechanism by which a T cell can broaden its specificity (Figure 1) (He et al., 2002; Welsh and Selin, 2002). However, this is only one potential mechanism of T cell cross-reactivity.

Through comparison of the unliganded and liganded crystal structures of a TCR, we can infer that conformational changes, small or large, allow the TCR to form a successful interaction with a pMHC (Garcia et al., 1998). Such conformational changes are often mediated by the TCR CDR3 loops. Their characteristic flexibility likely stems from their variable length and sequence, where longer loops containing multiple non-bulky aa residues, such as glycine, have fewer constraints to alleviate upon engaging the pMHC (Abergel and Claverie, 1991; Bentley and Mariuzza, 1996; Garcia et al., 1999; Hare et al., 1999; Reiser et al., 2002). Although the TCR makes this effort to accommodate the structure of its ligand, relatively few contacts between a TCR and its peptide appear necessary for a stable interaction; based on the solved crystal structures, a CD8 TCR generally only makes direct contact with positions 5, 7 and 8 of a 9-mer peptide sequence (Bjorkman, 1997; Rudolph and Wilson, 2002). In fact, a single aa residue, representing the primary TCR contact site, may be the only essential component shared among peptide ligands recognized by a given TCR (Evavold et al., 1995). The

large amount of space left between a TCR and its peptide-MHC is filled with water molecules that become equally important to the specificity of the interaction (Rudolph and Wilson, 2002).

The ability to accommodate various ligand structures through conformational change, the relatively few contacts with peptide, and the overall poor shape complementarity between a TCR and the peptide-MHC complex are all qualities of the interaction that facilitate T cell cross-reactivity (Garcia et al., 1998; Willcox et al., 1999). Crystal structures of the same human TCR bound to multiple different peptide ligands in the context of HLA-A2 provided the first clues as to mechanism behind a cross-reactive interaction, where most of the structural adjustments were mediated by the CDR3β loop, which contained three glycine residues (Ding et al., 1999). However, there were surprisingly few differences in the overall conformation of these crystal structures, which may be explained by the fact that each of the ligands differed by only 1 aa residue. The first crystal structures comparing a TCR engaged to two different ligands having no sequence similarity revealed a much larger structural adjustment, which was again mediated by a CDR3 loop, specifically CDR3α (Reiser et al., 2003). The authors suggested that these two peptide ligands had completely different structures, and their data supported a conformer hypothesis whereby an unliganded TCR is at equilibrium as a dynamic population of conformations until engaging a peptide ligand that will shift the equilibrium in favor of only one of those TCR conformations (Gagnon et al., 2005). Others have suggested that any initial structural dissimilarity between ligands is irrelevant because, upon interaction, all ligands are molded to fit a conserved

conformation necessary for specific TCR recognition (Lee et al., 2004).

Although the exact mechanism(s) behind a cross-reactive TCR interaction is largely unresolved, it is now accepted that a given T cell can interact with multiple different peptide ligands, with or without a high degree of sequence or structural similarity between them. One of the aims of this thesis research is to determine how frequently cross-reactive TCR interactions occur, and it will be interesting to see how many, if any, of these potential mechanisms of T cell cross-reactivity apply in our model system.

G. Epstein-Barr virus

To study CD8 T cell cross-reactivity and heterologous immunity, we chose EBV as our model system because of its high infection rate, its ability to induce an immune-mediated disease, and its large genome size, which increases its potential to encode multiple T cell epitopes that may elicit cross-reactive CD8 T cell responses. Thus, it is important to understand the biology of this virus and how it might activate a CD8 T cell response. EBV, also known as human herpesvirus 4 (HHV4), is a viral species of the Lymphocryptovirus genus and of the Gammaherpesvirinae subfamily that infects over 90% of the U. S. population. It has a large double-stranded DNA genome of 184kbp. EBV is released into and spread through saliva. Although epithelial cells, and possibly T cells and NK cells, can become infected in vivo and support viral replication during the lytic stage of the viral life cycle, EBV primarily infects resting naïve B cells found in the secondary lymphoid tissue of Waldeyer's tonsillar ring and uses CD21 and MHC Class II molecules as its receptor and co-receptor respectively (Thorley-Lawson and Gross, 2004;

Young and Rickinson, 2004). EBV likely exploits B cell biology to establish a life-long persistent infection within its host, transitioning through different transcriptional programs that appear to drive the B cell through its normal stages of differentiation (Figure 3) (Thorley-Lawson and Gross, 2004; Young and Rickinson, 2004). Upon infection of a naïve B cell, EBV enters its growth program, or type III latency stage, which appears to activate the cell to become a proliferating lymphoblast. At this time, the infected B cell does not encode any lytic viral proteins but can express epitopes derived from all 9 latent viral proteins on its surface and has the potential to activate both CD4 and CD8 T cells. Transition to the default program, or type II latency stage, appears to promote the survival of the lymphoblast and to support its differentiation into a memory B cell, in a process that resembles a germinal center reaction. This process involves the expression of only 3 of the latent viral proteins and, therefore, the number of T cell epitopes is reduced at this stage of the viral life cycle. Upon B cell differentiation, the virus transitions to its latency program, or type I latency stage, and remains at this stage for the lifetime of the memory B cell. Except for occasional EBNA-1 expression, the virus ceases all gene expression during this time and virtually escapes T cell recognition as the memory B cell circulates through the peripheral blood, however T cell responses directed at EBNA-1 have been identified (reviewed in (Munz, 2004)). It is not until the B cell is further activated by a non-EBV driven mechanism that it completes differentiation into an antibody-secreting plasma cell, and EBV once again initiates its replication cycle, or lytic phase. A memory B cell undergoing this final stage of differentiation has initiated viral replication, is located in the lymphoid tissue, and is

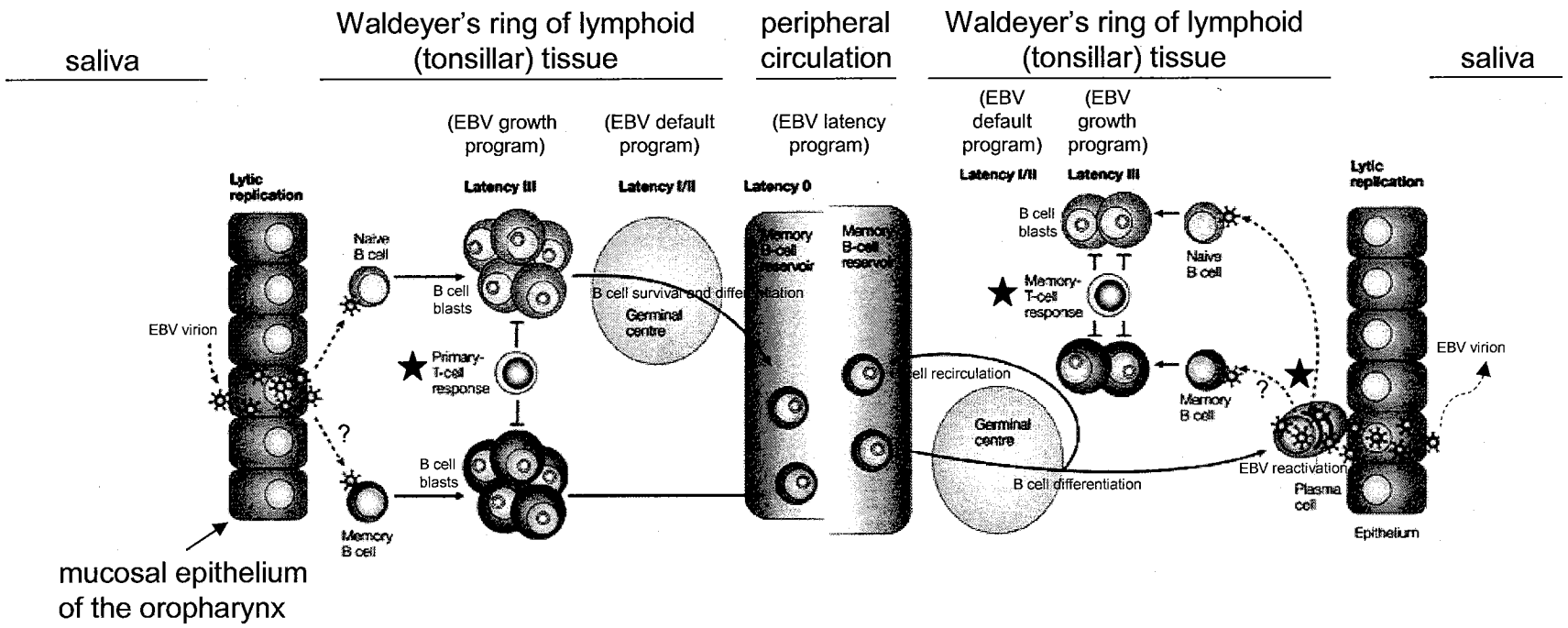


Figure 3. The putative lifecycle of EBV in the context of B cell biology. Points of interaction between virus-specific CD8 T cells and infected B cells are denoted by a red star. Direct EBV infection of memory B cells is currently controversial. This figure was adapted from Young et al. 2004 Nat Rev Cancer 4:757-768 and Thorley-Lawson et al. 2004 N Engl J Med 350:1328.

likely to be the major target of the EBV-specific CD8 T cell response.

H. CD8 T cell response to acute EBV infection

Although EBV is never completely eliminated from its host, there are several reasons to believe that CD8 T cells are largely responsible for controlling viral replication through the detection and destruction of infected host cells that express EBV-derived epitopes. The rapid reduction in virus shedding and in the proportion of latently infected B cells in the blood observed at convalescence implies effective T cell-mediated lysis of infected target cells (Rickinson and Kieff, 2001). Furthermore, T cell immunocompromised individuals often cannot effectively control a primary infection with EBV or lose their ability to control a pre-exisiting latent EBV infection, resulting in increased levels of virus shedding and increased numbers of infected B cells in the blood (Rickinson and Kieff, 2001). As a result, immunocompromised individuals can develop EBV-induced B cell lymphomas and, it is interesting to note, that immunotherapeutic attempts involving the transfer of polyclonal preparations of activated EBV-specific donor T cells frequently reversed tumor growth in the recipient and restored immune control over viral replication (Rickinson and Kieff, 2001).

The CD8 T cell response to acute EBV infection is detectable following a 2-7 week virus incubation period, coincident with a noticeable increase in the number of infected B cells (Iwatsuki et al., 2004). Most of what is known regarding the primary T cell response to EBV is based on studies of adolescents because they acquire primary EBV infections more frequently than adults and their immune response is greater in magnitude than that of children with primary EBV infection. These studies revealed that

the CD8 T cell response was directed against both latent and lytic EBV proteins and could comprise up to 47% of all CD8 T cells in the peripheral blood, as reported in some adolescents with infectious mononucleosis, an EBV-associated pathology that will be discussed separately (Callan et al., 1998; Catalina et al., 2001). While the primary response to latent proteins is delayed and of lower frequency than the primary response to lytic proteins, CD8 T cell reactivity has been detected against several latent proteins, most frequently the EBNA-3 proteins (Callan et al., 1998; Catalina et al., 2001; Rickinson and Moss, 1997; Steven et al., 1997; Tan et al., 1999). Those known to be restricted by HLA-A2 and the frequency range of primary T cells specific for them are noted in Table 1.

In addition to the viral proteins expressed at various points during the latent stages of the viral lifecycle, the lytic stage includes the expression of at least 60 viral proteins (Tierney et al., 1994). The CD8 T cell response to lytic viral proteins occurs earlier and is of a greater magnitude than the CD8 T cell response to latent viral proteins (Callan, 2003; Catalina et al., 2001). Immediate-early lytic proteins are transactivators, responsible for initiating the transcription of many cellular and viral genes, and derive many immunodominant CD8 T cell epitopes (Pudney et al., 2005). Early lytic proteins are largely involved in viral DNA synthesis and are also the source of several immunodominant epitopes. The late lytic proteins include the envelope glycoproteins and are less frequently the source of immunodominant epitopes. Primary CD8 T cell responses specific for lytic viral proteins, and which are restricted by HLA-A2, can be found in Table 1. During the primary response, EBV-specific T cells typically express

Table 1. HLA-A2-presented, EBV-derived, latent and lytic cycle epitopes and the frequency range of CD8 T cells specific for them.

A. Latent cycle epitope	aa sequence	Reference	% of A2+ responders*	**Primary T cell freq	**Memory T cell freq
BARFO	LLWAARPRL	(Kienzle et al., 1998)	0% (0%)$^{+}$	ND	ND
EBNA-2A$_{64-83}$	***	(Schmidt et al., 1991)	ND	ND	ND
EBNA-3A$_{596-604}$	SVRDRLARL	(Burrows et al., 1994)	10% (70%)$^{+}$	0.05-0.06%$^{+}$	0.05-0.4%$^{+}$
EBNA-3C$_{284-293}$	LLDFVRFMGV	(Kerr et al., 1996)	ND (60%)$^{+}$	ND	31-270 pCTL/10^{6} PBMC^{+}
LMP-1$_{125-133}$	YLLEMLWRL	(Khanna et al., 1998)	0% (0-70%)$^{+}$	ND	18-102 pCTL/10^{6} PBMC^{+}
LMP-1$_{159-167}$	YLQQNWWTL	(Khanna et al., 1998)	0% (14-55%)$^{+}$	ND	0.09-0.5%$^{+}$
LMP-2$_{329-337}$	LLWTLVVLL	(Lee et al., 1996)	0% (0%)$^{+}$	ND	ND
LMP-2$_{356-364}$	FLYALALLL	(Lautscham et al., 2003)	ND	ND	ND
LMP-2$_{426-434}$	CLGGLLTMV	(Lee et al., 1993)	11% (63-75%)^{o}	0.1%^{o}	0.05-0.4%^{o}

B. Lytic cycle epitope	aa sequence	Reference	% of A2+ responders*	**Primary T cell freq	**Memory T cell freq
BALF-4$_{276-284}$	FLDKGTYTL	(Pudney et al., 2005)	22% (ND)§	ND	ND
BALF-5	ND	(Pudney et al., 2005)	33% (ND)§	ND	ND
BBRF-1	ND	(Pudney et al., 2005)	11% (ND)§	ND	ND
BHLF-1	ND	(Pudney et al., 2005)	11% (ND)§	ND	ND
BMLF-1$_{280-288}$	GLCTLVAML	(Scotet et al., 1996) (Steven et al., 1996)	100% (91-100%)$^{+,o}$	0.08-47%$^{+,o}$	0.05-2.5%$^{+,o}$
BMRF-1$_{208-216}$	TLDYKPLSV	(Hislop et al., 2002)	100%(17-44%)^{o}	0.3-0.6%^{o}	0.05-1%^{o}
BRLF-1$_{109-117}$	YVLDHLIVV	(Saulquin et al., 2000)	100% (75-100%)^{o}	2.7-34%^{o}	0.05-2.4%^{o}
BZLF-1	FMVFLQTHI	(Stuber et al., 1995)	63% (12%)$^{+}$	0.01-1.3%$^{+}$	0.01-0.15%$^{+}$
GP350$_{863-871}$	VLQWASLAV	(Khanna et al., 1999)	25% (0%)$^{+}$	ND	ND
GP85$_{225-233}$	SLVIVTTFV	(Khanna et al., 1999)	50% (25%)$^{+}$	ND	ND
GP85$_{420-428}$	TLFIGSHVV	(Khanna et al., 1999)	50% (15%)$^{+}$	ND	ND
GP85$_{542-550}$	LMIIPLINV	(Khanna et al., 1999)	50% (33-50%)$^{+}$	ND	20-22 pCTL/10^{6} PBMC^{+}

* Percent of HLA-A2+ people with T cells of this specificity during the acute phase of EBV infection or, as shown in parenthesis, during the persistent phase of EBV infection, ** frequency is of total CD8 T cells unless otherwise stated, ***QLSDTPLIPLTIFVGENTGV, $^{+}$ based on IFNγ production as reported by (Bharadwaj et al., 2001; Catalina et al., 2001), ^{o} based on tetramer staining reported by (Hislop et al., 2002), § based on specific lysis of target cells by T cell clones reported by (Pudney et al., 2005), ND: not sufficiently determined or not reported

activation markers such as CD45R0, CD38, HLA-DR, CD69, CD244 (or 2B4), and downregulate the expression of lymphoid homing receptors such as CD62L and CCR7 (Callan et al., 1998; Catalina et al., 2002; Williams et al., 2004). This is the typical phenotype of most activated virus-specific T cells, an indication that they are primed and able to survey peripheral tissues for the presence of antigen. Studies have demonstrated that activated EBV-specific T cells have immediate effector functions using ex vivo cytotoxicity assays and intracellular stains for perforin, IFNγ and MIP-1β (Callan et al., 2000; Catalina et al., 2002). The phenotype and function of activated EBV-specific T cells will be important for comparison to the phenotype and function of memory T cells during this acute infection in order to assess whether any productive cross-reactive interactions have occurred.

I. Infectious mononucleosis

Two qualities of the primary immune response to EBV that make it somewhat unusual compared to that directed at other viruses are the extent of immune activation and the large magnitude of the T cell response, both of which are thought to contribute to the age-related T cell-mediated pathology known as infectious mononucleosis (IM) (Silins et al., 2001; Williams et al., 2004). Primary EBV infection during childhood is often sub-clinical and goes unnoticed. Therefore, little is known about the kinetics and specificity of the primary immune response to a childhood infection. Alternatively, primary EBV infection during adolescence frequently presents as IM. EBV-associated IM is confirmed by a serological test for the presence of viral capsid antigen-specific IgM antibody, and patients typically present with fever, pharyngitis and lymphadenopathy;

Splenomegaly and hepatomegaly can occur, but are often missed in the absence of ultrasound examination (Ebell, 2004). Older adults with acute EBV infection more commonly have complications associated with IM, including hepatomegaly, jaundice and even fulminant hepatitis (Auwaerter, 1999; Ebell, 2004).

IM is an immune-mediated pathology, the symptoms of which have been attributed to the hyperexpansion of activated CD8 T cells (lymphocytosis) that secrete a variety of inflammatory and antiviral cytokines, likely contributing to the elevated levels of IL-2, IL-6, TNFα, IFNγ and MIP-1β previously detected in patient sera and tonsils (Foss et al., 1994; Iwatsuki et al., 2004; Nakayama et al., 2004). One of the criteria for IM diagnosis is the presence of at least 50% lymphocytes and at least 10% atypical lymphocytes in the peripheral blood, of which the majority are EBV-specific CD8 T cells (Ebell, 2004; Wood and Frenkel, 1967). The degree of lymphoproliferation and the extent of immune activation, marked by the prolonged expression of CD244 on CD8 T cells, have both correlated with the presence and severity of IM symptoms (Silins et al., 2001; Williams et al., 2004). CD244, or 2B4, is a component of the SAP/SLAM lymphocyte activation pathway and its expression has correlated with the acquisition of T cell effector functions, such as granzyme B and perforin expression and rapid IFNγ production (Speiser et al., 2001). It is still unclear what causes such a high degree of lymphoproliferation and immune activation during the acute EBV response. Two possible explanations put forth in the field are 1) a high viral load and 2) the additional activation of memory CD8 T cells, either through a TCR-mediated or bystander mechanism (Rickinson and Kieff, 2001; Rickinson and Moss, 1997; Silins et al., 2001).

Evidence against the idea that a high viral load is the cause of the exaggerated T cell response includes a study showing similar levels of virus in asymptomatic and symptomatic individuals during acute EBV infection and at least two studies demonstrating that antiviral drugs, such as acyclovir, reduced viral load but had no affect on the symptoms of IM (Andersson et al., 1987; Torre and Tambini, 1999). Thus, this thesis research is designed to test the theory that EBV-induces the activation of cross-reactive memory CD8 T cells through a TCR-mediated mechanism.

J. CD8 T cell response to persistent EBV infection

Although EBV establishes a persistent infection, the massive CD8 T cell response typical in IM patients is eventually downregulated during convalescence. Many T cells are deleted, but some survive and form a "memory" T cell population, which appears necessary to keep the virus under control by preventing or suppressing a productive re-activation of viral replication (Rickinson and Kieff, 2001). The frequency of CD8 T cells specific to any one EBV-derived epitope may still constitute a large proportion of the total memory T cell pool, comprising up to 5.5% of CD8 T cells in the peripheral blood as determined with MHC Class I tetramers (Callan, 2003). The frequency range of memory T cells specific for the known HLA-A2-restricted epitopes are presented in Table 1. Early comparisons of the primary and memory CD8 T cell responses of individual patients revealed differences in the relative frequencies of each epitope-specific response during these two distinct phases of the immune response. For instance, one study reported that the response to immunodominant epitopes was more extensively downregulated than that to subdominant epitopes during the contraction phase of the

primary T cell response, resulting in an altered epitope hierarchy in memory (Steven et al., 1996). However, there were many caveats associated with this study, which relied on in vitro stimulated T cell lines and limiting dilution assays that may not equally support the growth of all T cell clones and which did not assess T cell reactivity to lytic epitopes. A more recent comparison of the clonal composition of primary and memory EBV-specific responses used ex vivo tetramer+ T cell populations and monoclonal antibodies to assess their Vβ usage, which more accurately demonstrated that the most dominant Vβ-specific T cell populations, of the two epitope-specific responses examined, were heavily culled during the establishment of memory (Callan et al., 2000). While the same dominant epitope-specific, Vβ-defined, T cell clones were detected in the memory T cell pool, they were present at lower frequencies. It has been postulated that the extent of downregulation of an epitope-specific T cell response has to do with the limited lifespan of the T cell clones comprising that response, a concept referred to as T cell senescence (Davenport et al., 2002). According to this theory, a higher rate of T cell proliferation, as is associated with immunodominant responses, will cause a lower rate of T cell survival into memory. This concept offers one explanation for why T cell responses to latent epitopes are often more pronounced in the memory phase because they are often subdominant to the T cell responses directed at lytic epitopes during the primary phase and, therefore, undergo less proliferation during that time (Callan, 2003; Callan et al., 1998; Catalina et al., 2001). While an intriguing theory, this clearly does not appear to occur during well-characterized non-persistent infections, such as LCMV-Armstrong, where the epitope-specific hierarchy present during the acute phase of the infection is

maintained into memory. Thus if the theory holds true, T cell senescence may more often be associated with persistent viral infections, where antigen concentrations remain high or increase periodically during episodes of viral replication.

K. Features of the EBV(BMLF1)-specific TCR repertoire

The T cell response to the early lytic epitope EBV-BMLF1$_{280\text{-}288}$ is often immunodominant during the primary phase of the immune response (Table 1). The TCR repertoire that recognizes BMLF1, among all other known EBV-derived epitopes, has been the best characterized and has been described as oligoclonal. However, this repertoire has characteristics of both a diverse and a restricted repertoire. The repertoire is diverse for two reasons: 1) the dominant Vβ family, as well as the clonotypic composition of that family, varies between individuals and can be referred to as the private specificity of the BMLF1-specific repertoire, and 2) multiple subdominant Vβ families are often used by each individual (Annels et al., 2000; Callan et al., 2000; Lim et al., 2000). These observations imply that multiple different TCR structures can interact with BMLF1. There are also two features of this repertoire that restrict it: 1) despite individual variation in the combination of Vβ families used, each individual's repertoire often includes one or more of 4 common Vβ families (Vβ2, 4, 16, 22) and 1-2 common Vα families (Vα2.3, 15), which can be referred to as the public specificity of the BMLF1-specific repertoire, and 2) each Vβ family is frequently comprised of very few clones (Annels et al., 2000; Callan et al., 2000; Cohen et al., 2002; Lim et al., 2000). One of the most conserved elements of the BMLF1-specific TCR repertoire is the usage of Vα15 because it can pair with 3 of the 4 common Vβ families, suggesting that the α-

chain may contribute more than the β-chain to the interaction with BMLF1 (Lim et al., 2000). Clones expressing Vα15 have conserved, usually germline encoded, CDR3α motifs that vary depending on which Vβ family is expressed (Annels et al., 2000; Lim et al., 2000). Each of four frequently expressed Vβ families (Vβ2, 4, 16, 22) is commonly associated with a particular Jβ family and uses a conserved CDR3β motif, which often includes non-germline encoded N-additions (Table 2) (Annels et al., 2000; Lim et al., 2000). The one outstanding feature of these BMLF1-specific CDR3β motifs was the presence of one or more glycine residues, however, the significance of this is unclear (Annels et al., 2000; Cohen et al., 2002; Lim et al., 2000).

Analyses of the ex vivo BMLF1-specific TCR repertoire have demonstrated that while the clonal composition of given Vβ family appeared stable over time, the Vβ hierarchy changed during the establishment of memory, resulting in a decreased proportion of T cells expressing a Vβ family that once dominated the primary epitope-specific response (Callan et al., 2000). One interpretation of these data is that the dominant Vβ-specific response was heavily culled during the contraction phase of the immune response, but not to the extent that it was completely deleted (Callan et al., 2000). T cell senescence is one possible explanation for this result since BMLF1 is thought to be an immunodominant epitope during the acute phase of EBV infection and the responding T cells are likely to proliferate extensively during this time, many of which could reach the end of their finite lifespan (Davenport et al., 2002). Alterations in the clonal hierarchy of the BMLF1-specific response were only observed in one study, which relied on the long-term in vitro proliferation of T cells that can bias the results

Table 2. The public specificity of a BMLF1-specific TCR repertoire.

Vβ family	Jβ family	CDR3β sequence	Vα family	Jα family	CDR3α sequence
2	1.2	SA-R**D*TGN**GY-TF	15	varies	AE-**D***NARL-MF
	1.3	SA-R***GV**GNTI-YF			
4	1.4	SV-**GTGG**TNEKL-FF	15	varies	AE-SIGKL-IF
16	varies	AS-SQ**SPGG**TQ-YF	15	3	AE-YSSASKI-IF
22	2.1, 2.2	AS-S***G*V*P**GEL-FF	2.3	12[§]	VV-N**G**MDSSYKL-IF

This table was adapted from (Lim et al., 2000), and has been confirmed by data reported in (Annels et al., 2000; Cohen et al., 2002). All V family nomenclature is based on (Arden et al., 1995), [§]Jα nomenclature according to the Immunogenetics database. Bolded residues are non-germline encoded, and include the Dβ region in the case of CDR3β.

(Annels et al., 2000). However, one interesting finding came from that study, which was that amino acids in positions 4 and 6 of the BMLF1 peptide sequence most likely make contact with the TCR based on alanine substitution experiments and, overall, Vβ22/Vα2+ T cell clones were more adaptable to variant BMLF1 peptide ligands than were other clones, such as those expressing Vβ2/Vα15 (Annels et al., 2000).

L. Influenza A virus

The other virus-specific T cell response investigated in this thesis work was that specific for influenza A virus (IV). Most of the world's population has been infected with IV by a young age and, therefore, have virus-specific memory CD8 T cells. This gave us the opportunity to investigate the cross-reactive potential of a memory T cell population that is shared among many different individuals with otherwise unique histories of infection. Multiple studies investigating the presence of IV-specific memory T cells have reported between 55-100% of adults have previously been exposed to IV (Boon et al., 2002; He et al., 2003; Lalvani et al., 1997). IV-specific memory T cells can comprise 0.06-1.6% of the CD8 T cell population found in the peripheral blood, as determined by stimulating PBMC with either live virus (H3N2, Resvir-9) or inactivated allantoic fluid from infected eggs (H3N2, Sydney and H1N1, Beijing) and then staining extracellularly for CD8 and intracellularly for the production of IFNγ (Boon et al., 2002; He et al., 2003).

Although both are common human viruses, IV is very different from EBV. Influenza A viruses represent one genera of the Orthomyxoviridae family (Wright and Webster, 2001). Co-infection with multiple sub-types of IV promotes gene reassortment,

known as antigenic shift, and the emergence of new sub-types. IV sub-types are defined by the HA and NA genes expressed by the virus. HA and NA are two of 10 known viral proteins. They are found on the surface of the virion and are the major targets of the humoral immune response. Hence these two proteins often undergo antigenic drift, where minor changes in their immunogenicity allow certain virus strains to escape detection by neutralizing antibodies. Thus, the existence of multiple sub-types and strains, to which a host has insufficient humoral immunity, leaves a host susceptible to repeat, but non-persistent, infections with IV.

IV is spread via an aerosol route and, thus, the primary site of viral replication is the respiratory tract, predominantly in the resident epithelial and mononuclear cells (Wright and Webster, 2001). The incubation period is approximately 3 days, when there is a peak in viral load, and virion shedding stops between days 6-8 post-infection. Clinical signs and symptoms display tremendous variability, ranging from an asymptomatic infection to death, and appear to depend heavily on the age of the individual and the presence of any underlying medical conditions. The symptoms of an uncomplicated infection include headache, fever, chills, cough, myalgia, and malaise, all of which usually resolve within 1-2 weeks. Increased morbidity often occurs in the very young (<5 years of age) and the very old (>65 years of age), presumably due to the absence or waning of pre-existing immunity to IV, and is commonly associated with severe viral pneumonia.

M. Primary and memory CD8 T cell responses to IV

Research on natural acute IV infection is lacking largely because patients are

rarely admitted to hospitals or visit outpatient clinics and, when they do, diagnostic tests for IV are rarely performed. An increase in the number of CD8 T cells can be found in the peripheral blood between days 6-14 post-infection and returns to baseline between days 21-28, but the average frequencies and activation phenotype of epitope-specific T cells during this phase of the immune response have not been extensively studied (Wright and Webster, 2001).

Each IV infection is transient, and the cellular immune response likely contributes to viral clearance. The CD8 T cell response is known to target almost all viral proteins: HA, M1, M2, NS1, NP, PB1, and PB2 (Gotch et al., 1987; Jameson et al., 1998; Man et al., 1995). M1, or matrix protein, underlies the lipid bilayer of the virion. It is the most abundant viral protein and is a target of the immunodominant response in most individuals (Wright and Webster, 2001). The highly conserved sequence of M1 among different viral sub-types and strains likely explains the maintenance of M1-specific memory T cells over time (Boon et al., 2002; Naumov et al., 2003; Prevost-Blondel et al., 1997). In addition to the wide variety of epitopes derived from IV, a wide variety of HLA alleles are used to present these epitopes to IV-specific CD8 T cells (Jameson et al., 1998). All individuals who express HLA-A*0201 use this allele to present IV-specific peptides, however, there is evidence to suggest that HLA-B alleles, such as B*3501 and B*2701, often elicit a more dominant CD8 T cell response (Boon et al., 2004). HLA-A*0201 molecules present an immunodominant epitope, $M1_{58-66}$, and 78-100% of individuals expressing this allele are reported to have memory T cells specific for this epitope (Bednarek et al., 1991; Gotch et al., 1987; Lalvani et al., 1997; Lawson et al.,

2001; Lehner et al., 1995; Pittet et al., 2001). In memory, the frequency of $M1_{58-66}$-specific CD8 T cells varies among HLA-A2+ individuals, ranging from 1/250,000 to 1/2125 PBMC, and has been estimated to average 0.13% of CD8 T cells in the blood (Jameson et al., 1998; Lalvani et al., 1997; Lehner et al., 1995; Pittet et al., 2001; Prevost-Blondel et al., 1997). HLA-A*0201 molecules also present several other epitopes, which are presumably subdominant due to the decreased ability to detect T cell responses directed against these epitopes among individuals (Table 3). However, a cross-reactive stimulation may result in the expansion of one of these otherwise subdominant IV-specific memory T cell populations, altering the resting-state epitope-specific hierarchy.

N. Features of the (IV)M1-specific TCR repertoire

This thesis will focus on the TCR repertoire directed towards the immunodominant, HLA-A2-presented, IV $M1_{58-66}$ epitope. Point mutations in the M1 peptide initially determined that the central portion of the peptide was important for its presentation and subsequent recognition by M1-specific CD8 T cells. Positions 4-6 were thought to face the MHC Class I molecule, while positions 3, 7 and 8 of the peptide sequence were thought to face the TCR (Gotch et al., 1988). Once the crystal structure of the M1:HLA-A2 complex was solved, it was evident that M1 was unusual in its lack of prominent aa side chains directed towards the TCR (Madden et al., 1993). It is possible that the unique structure of the M1 peptide in the context of HLA-A2 plays a role in the selection of an immunodominant M1-specific TCR structure, expressing Vβ17/Jβ2.7 and Vα10.2/Jα42 and having the conserved CDR3β motif *RS* and a CDR3α loop that is

Table 3. HLA-A2-presented, IV-derived, epitopes and the frequency range of CD8 T cells specific for them.

Epitope	aa sequence	Reference	% of A2+ responders*	Memory T cell freq
$M1_{58-66}$	GILGFVFTL	(Gotch et al., 1987)	75-100%$^{+}$	1/250,000-1/2125 PBMC or 0.13% of CD8 T cells^{+}
$M1_{59-68}$	ILGFVFTLTV	(Gianfrani et al., 2000)	67%	ND
NA_{75-84}	SLCPIRGWAI	(Gianfrani et al., 2000)	17%	ND
$NA_{213-221}$	CVNGSCFTV	(Wedemeyer et al., 2001)	ND	ND
NP_{85-94}	KLGEFYNQMM	(Robbins et al., 1989)	ND	ND
$NS1_{122-130}$	AIMDKNIIL	(Man et al., 1995)	ND	<1/500,000 PBMC^{o}
PA_{46-54}	FMYSDFHFI	(Gianfrani et al., 2000)	33%	ND
$PA_{225-233}$	SLENFRAYV	(Gianfrani et al., 2000)	33%	ND
$PB1_{413-421}$	NMLSTVLGV	(Gianfrani et al., 2000)	33%	ND

* Percent of HLA-A2+ people in a resting memory state with T cells of this specificity, $^{+}$ (Jameson et al., 1998; Lalvani et al., 1997; Lawson et al., 2001; Lehner et al., 1995; Pittet et al., 2001; Prevost-Blondel et al., 1997), ^{o} (Jameson et al., 1998), ND: not sufficiently determined or not reported

extended by multiple glycine residues (Lawson et al., 2001; Lehner et al., 1995; Moss et al., 1991; Naumov et al., 1998; Naumov et al., 2005; Stewart-Jones et al., 2003) (Naumov et al., manuscript in preparation).

The full crystal structure of this immunodominant TCR (*JM22*) bound to the M1:HLA-A2 complex revealed some unusual characteristics of this interaction compared to most of the 19 other structures solved to date (Stewart-Jones et al., 2003). First, the orientation of the TCR over the pMHC was more orthogonal than the typical diagonal orientation of other known structures. The authors suggested that the CDR1β and CDR2β regions of Vβ17 directed this orientation. Second, the interaction between the TCR and pMHC is biased toward the TCR β-chain, providing 67% of the contact region, while other structures often use both chains equally or have an α-chain bias. The CDR3β loop is centered over the M1 peptide, and the authors suggest it is responsible for providing the specificity for a high avidity interaction. While the α-chain provides no hydrogen bonds to the interaction, it does make direct, but weak, contact with positions 4 and 5 of the M1 sequence and contributes to the overall stability of the complex. Third, due to the lack of prominent side chains at the interface, water molecules are forced to make a vital contribution to the shape complementarity between the TCR and the pMHC. Overall, it was reported that four key interactions conferred the specificity of the *JM22* TCR for M1:HLA-A2, and those interactions are diagrammed in Figure 4 (Stewart-Jones et al., 2003).

Despite this dominant selection for TCRs expressing Vβ17 and Vα10.2, the M1-specific TCR repertoire can still be described as polyclonal. Multiple Vβ17+ T cell

A.

B.

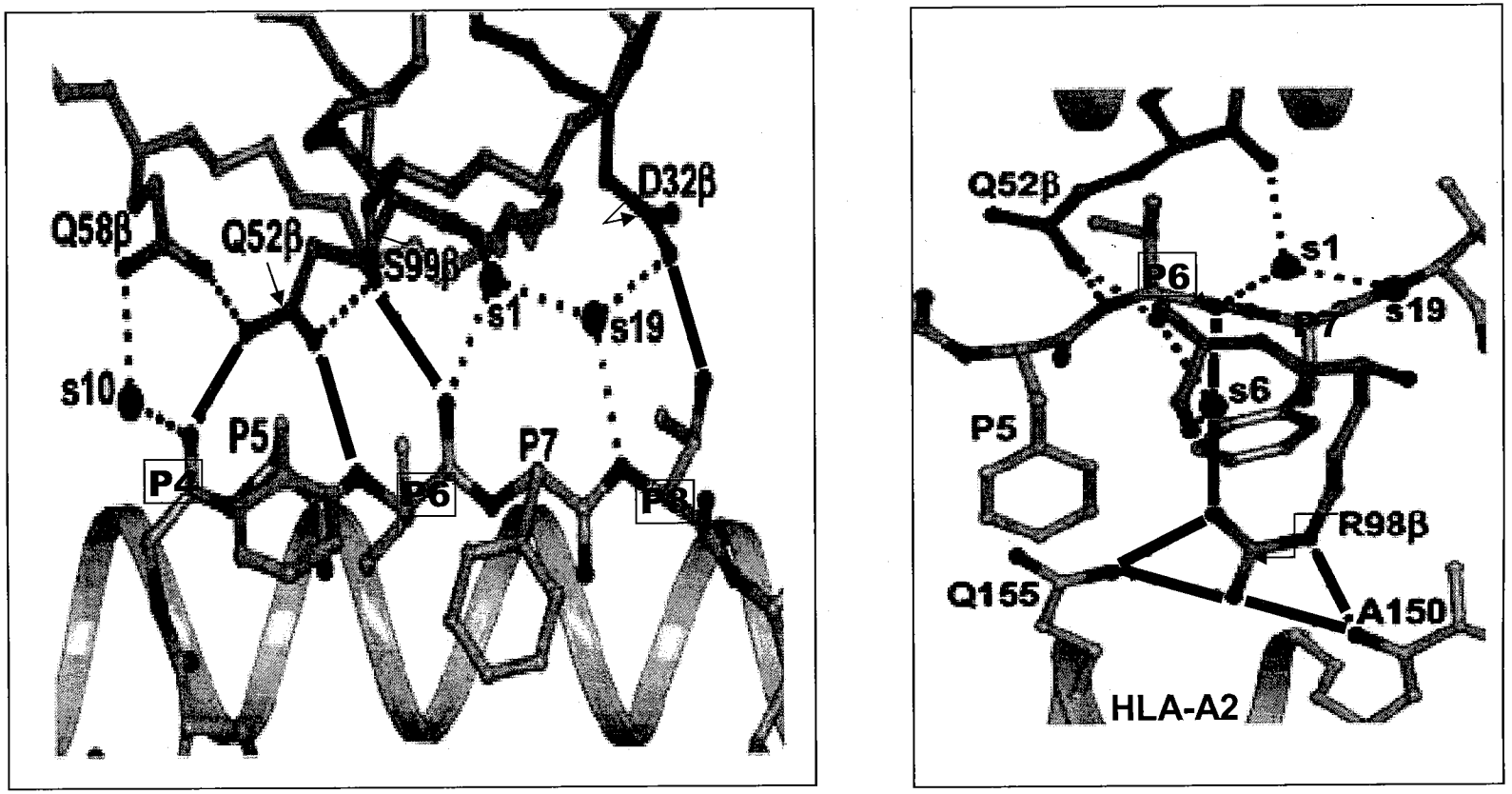


Figure 4. Key interactions that explain the public usage of the *JM22* TCR β-chain for engaging the M1/ HLA-A2 complex. The four key contact points on the TCR are (A) D32 (CDR1β, purple), Q52 (CDR2β, orange), S99 (CDR3β, cyan) and (B) R98 (CDR3β, cyan). The M1 epitope (GILGFVFTL) is depicted in yellow and the position of the aa residue involved in the interaction is boxed in pink. The hydrogen bonds mediating the pertinent interactions are highlighted in pink. A water molecule, depicted as the red sphere s6, bridges the bond between R98 and P6. These diagrams were taken from Stewart-Jones et al. 2003 Nat Immunol 4:657-663.

clones exist, based on their unique nucleotide sequence, that encode the characteristic *RS* CDR3β motif (Naumov et al., 1998; Naumov et al., 2005; Naumov et al., 2003). There are also several Vβ17+ T cell clones that express alternative Jβ families and thus express variations in the length and sequence of the CDR3β loop (Naumov et al., 2005; Naumov et al., 2003). Furthermore, some M1-specific T cell clones express alternative Vβ and Vα families, although these clones are present at much lower frequencies (Lawson et al., 2001; Naumov et al., 2005) (Naumov et al., manuscript in preparation). By maintaining a vast distribution of M1-specific T cell clones, the majority of which remain at a relatively low frequency, the immune system is better able to adapt to any changes in the concentration and sequence of the M1 peptide ligand over time (Naumov et al., 2005; Naumov et al., 2003).

O. Thesis objective

EBV has a large genome with the capacity to encode a multitude of T cell epitopes simultaneously throughout the life of its host, many of which could be recognized by memory CD8 T cells specific for other pathogens. Furthermore, primary EBV infection is frequently asymptomatic in children but in adolescents and young adults infection often results in a T cell-mediated disease pathology known as infectious mononucleosis, the clinical signs and symptoms of which can range from mild to severe. Since exposure to pathogens generally increases with age, it is likely that adolescents and young adults have a greater proportion of cross-reactive CD8 T cells in their memory T cell pools than do children. **Thus, our hypothesis was that cross-reactive memory CD8 T cells specific for previously encountered pathogens are activated during acute**

EBV infection, and that these cross-reactive CD8 T cell responses are, at least in part, responsible for the development of infectious mononucleosis. My thesis work is presented in three parts:

Chapter III: The identification of cross-reactive CD8 T cell responses involving EBV-derived, HLA-A2-presented, epitopes.

Chapter IV: Cross-reactive CD8 T cells specific for EBV-BMLF1$_{280-288}$ and IV-M1$_{58-66}$ contribute to the lymphoproliferation in EBV-associated infectious mononucleosis.

- **a.** What is the frequency of this cross-reactive response among individuals?
- **b.** What are the effector functions that result from this cross-reactive interaction?
- **c.** Does this cross-reactive response correlate with the severity of IM?

Chapter V: The clonal composition of the cross-reactive CD8 T cell response with specificity for EBV-BMLF1$_{280-288}$ and IV-M1$_{58-66}$: Features of a cross-reactive TCR repertoire.

- **a.** What is the structure(s) of this cross-reactive TCR and what features distinguish it from a non-crossreactive TCR?
- **b.** How is the cross-reactive repertoire organized? Does it have features of a public or private repertoire? Is it comprised of a narrow or broad collection of cross-reactive clones?

Increased disease severity among young adult and adult populations compared to children has been observed following infection with EBV and other human viruses, such as measles, mumps, and varicella-zoster viruses. In addition to age, there are many factors that can contribute to variations in disease severity, including genetics, virus strain and infecting dose, and the presence of underlying medical conditions that cause an immunocompromised state. This thesis examines the influence of an individual's history of infection and, in particular, cross-reactive memory CD8 T cell responses on the development and severity of infectious mononucleosis associated with EBV infection.

CHAPTER II:

MATERIALS AND METHODS

A. Human subjects

Influenza A virus-immune patients with acute EBV infection were between the ages of 18-23 and were volunteers from the University of Massachusetts (UMass) Student Health Services at Amherst, MA. HLA-typing was performed using the Lymphotype Class I system (Biotest Diagnostics, Denville, NJ) and an Olerup SSP kit (GenoVision, West Chester, PA). Acute EBV infection was confirmed by a monospot test and the detection of capsid-specific IgM in patient sera. Positive staining with HLA-A2-tetramers loaded with influenza-M1 was used as an indication that these individuals had been exposed to influenza A virus in the past. Patients provided up to eight blood samples (50 ml each) starting at presentation with IM (Day 0), then weekly for the following 6 weeks, and then again at 1 year. When possible, the severity of the clinical signs and symptoms of IM at the time of the visit were graded on a scale of 1 (mild) to 5 (severe).

Healthy donors between the ages of 24-60 were volunteers from the research community at UMass Medical School (Worcester, MA). HLA status and immunity to EBV and influenza A virus were assessed using monoclonal antibody (BB7.2, Becton Dickinson, San Diego, CA) and tetramer stains, respectively. Previous exposure to EBV was confirmed by the detection of capsid-specific IgG in donor sera. Donors provided up to three blood samples (60 ml each). This study was approved by the Human Studies Committee at UMass Medical School.

B. PBMC isolation

Whole blood was diluted 1:2 with Hank's balanced salt solution (Gibco, Carlsbad, CA) and 20-25 ml was layered over 15 ml of Ficoll-Paque Plus (Amersham Bioscience, Uppsala, Sweden) and subsequently spun at 360 g for 30 min with no brake. The PBMC layer was washed three times with the Hank's solution, spun for 10 min with brake, first at 320 g and then at 240 g for all subsequent spins.

C. CD8 T cell isolation

PBMC were passed through nylon mesh, counted, and resuspended in magnet buffer before they were stained with anti-CD8 microbeads (Miltenyi Biotech, Auburn, CA) according to the manufacturer's recommendation. Positive selection of CD8+ lymphocytes was performed using the Miltenyi Biotech MACS system.

D. CD8 T cell line cultivation

T2 cells (ATCC #CRL-1992) served as the antigen presenting cells after being pulsed with 1 μM of peptide for a minimum of 3 hours at 37 °C, irradiated with 3000 RAD, and washed of free peptide. CD8+ lymphocytes were plated at 2.5 x 10^5 per ml together with the T2 cells at 5 x 10^4 per ml in 4 ml total volume per well of a 12-well plate. T cell lines were fed media [AIM-V (Gibco) supplemented with 14% human AB serum (Nabi, Miami, FL or Gemini, Woodland, CA), 16% MLA-144 supernatant (Rabin et al., 1981), 10 U/ml rIL-2 (Becton Dickinson), 1% L-glutamine (Gibco), 0.5% β-mercaptoethanol (Sigma, St Louis, MO), 1% HEPES (Hyclone, Logan, UT)] every 3-4 days and were re-stimulated with fresh T2 cells weekly.

E. Peptides

The following peptides were synthesized to >90% purity by Biosource (Camarillo, CA): EBV-BMLF1$_{280-288}$ (GLCTLVAML), EBV-LMP2$_{329-337}$ (LLWTLVVLL), EBV-BRLF1$_{109-117}$ (YVLDHLIVV), EBV-EBNA3A$_{596-604}$ (SVRDRLARL), IV-M1$_{58-66}$ (GILGFVFTL), IV-NP$_{85-94}$ (KLGEFYNQMM), tyrosinase (YMNGTMSQV), HIV-gag$_{77-85}$ (SLYNTVATL). Peptide stocks used to pulse T2 cells were kept at 0.1 mg/ml in sterile water. Peptide stocks used for stimulation in intracellular assays were kept at 1 mg/ml in DMSO.

F. Tetramers

A detailed description of the protocol used by the tetramer facility at UMass Medical School has been previously published (Catalina et al., 2001). Tetramers were assembled using the above peptide sequences for EBV-BMLF1 and influenza-M1 and were conjugated to phycoerythrin (Sigma), allophycocyanin (Caltag, Burlingame, CA), or Quantum Red (Sigma). Tetramers assembled with HIV-gag or tyrosinase (Immunomics, San Diego, CA) were used as negative controls, and non-specific staining was never observed.

G. Extracellular staining

Freshly isolated or cultured T cells were plated at 10^6 per well and washed with FACS buffer (PBS, 2% FCS, 1% sodium azide). Tetramers were incubated at room temperature for a total of 40 min. In experiments where cells were co-stained with tetramers and additional surface markers, T cells were pre-incubated with tetramers for 20 min then allowed to incubate with all other antibodies for an additional 20 min at

room temperature. Cells were washed and fixed with FACS Lysing Solution (Becton Dickinson), incubating at room temperature for 5 min before finally being resuspended in FACS buffer. All TCR Vβ-specific antibodies were purchased from Immunotech (Marseille Cedex, France) and all other antibodies were purchased from Becton Dickinson. A FACS Calibur (Becton Dickinson) was used for most data acquisition, and all data analysis was completed using Flowjo software (Tree Star Inc., Ashland, OR). In cases where T cells could co-stain with more than one tetramer or antibody, fluorescence compensation was attained using single-color control samples prior to data acquisition to ensure that co-staining was specific and not the result of the fluorescence of one fluorochrome bleeding into the improper channel of the FACS Calibur. Under circumstances where fluorescence compensation could not be attained, co-staining was not assessed, and this has been noted in the applicable figures.

The total number of antigen-specific CD8 T cells per ml of blood was determined using the following calculation: (ex vivo % of tetramer+ cells/CD8) x (ex vivo % of CD8+ cells/PBMC) x (total number of PBMC isolated from the blood) divided by the total volume (ml) of blood prepared.

H. Intracellular staining

Except in peptide titration experiments where the concentration varied, T cells incubated with a standard 5 μM concentration of peptide in media for 5 hours at 37 °C in the presence of brefeldin A. When combined with extracellular staining, the same peptide stimulation protocol was used and T cells were then washed in FACS buffer and stained with tetramer for a total of 40 min at room temperature. T cells were washed and

then fixed and permeabilized using Cytofix/Cytoperm (Becton Dickinson) according to the manufacturer's instructions. The following monoclonal antibodies were used: anti-IFNγ (0.2 μg clone B27, Becton Dickinson), anti-MIP-1β (0.2 μg clone D21-1351, Becton Dickinson), and anti-TNFα (mAb11, eBioscience). The appropriate isotype control antibodies did not stain positive. Data acquisition and analyses were performed as described above.

I. CD8 T cell sorting

Freshly isolated or cultured T cells were stained extracellularly as described above using 2% FCS/PBS. T cell populations were sorted using the Mo-Flo cell sorter (DakoCytomation, Carpinteria, CA) and collected in T cell line media. Single T cells were sorted using a FACS Vantage (Becton Dickinson) and collected in 96-well plates containing feeder cells and T cell line media.

J. Short-term CD8 T cell cloning

Double tetramer-positive T cells were sorted at 1 cell per well of a 96-well plate. In addition, each well contained 10^5 irradiated donor-specific CD4+ T cell blasts and 2 x 10^3 of a 1:1 mixture of irradiated T2 cells pulsed with BMLF1 or M1 peptides. Clones were fed fresh media every 3-4 days, re-stimulated with T2 cells at day 7, and their specificity tested on day 14.

K. ^{51}Chromium release assay

K562 (Britten et al., 2002) and cos-7 cell lines stably transfected with HLA-A2.1 both worked as target cells, pulsed with 100 μM of peptide for 1 hour at 37 °C and then for an additional hour with 100 μCi ^{51}Cr per 10^6 cells. Target cells were washed three

times and plated at 5 x 10^3 per well of a 96-well plate. Day 14 T cell clones were tested using split-well analysis; 30 µl of each clone were loaded per well per target cell type. Spontaneous ^{51}Cr release was measured upon incubating target cells with 30 µl of irradiated feeder cells alone, which were cultured in parallel with the T cell clones for use as negative control cultures. Plates incubated 8 hours at 37 °C and the supernatants were harvested and counted using a MicroBeta TriLux scintillation counter (Perkin Elmer, Wellesley, MA). Any experimental value greater than three standard deviations above the average spontaneous release value was considered a positive result.

L. IFNγ ELIspot assay

Our protocol was adapted from a previously published method (Britten et al., 2002) using Mabtech (Mariemont, OH) reagents. Briefly, primary anti-IFNγ (mAb 1-D1K) was loaded onto Multiscreen-IP filter plates (Millipore, Billerica, MA) at 10 µg/ml and incubated overnight at 4 °C. The plates were then washed and blocked with 10% FCS/RPMI for 1 hour at 37 °C. K562/HLA-A2.1 or cos-7/HLA-A2.1 cell lines served as the antigen-presenting cells (APC), pulsed with 50 µM of peptide for 3 hours at 37 °C and washed of free peptide. These APCs were loaded at 10^5 per well. Day 14 T cell clones were tested using split-well analysis, whereby 5 µl of each clone were loaded per well per APC type. Negative control wells consisted of APCs plus 5 µl of irradiated feeder cells alone, which were cultured in parallel with the T cell clones. Plates incubated at 37 °C for 20 hours, were washed, and loaded with 2 µg/ml of the secondary, biotinylated, anti-IFNγ (mAb 7-B6-1). Following a 3 hour incubation at room temperature, plates were washed and loaded with streptavidin-horseradish peroxidase,

diluted 1:100, and incubated for 2 more hours at room temperature. Nova Red Substrate (Vector Labs, Burlingame, CA) was added to the plates according to the manufacturer's instructions and incubated for 40 min at room temperature. Plates were washed and left to air dry before being read manually. Negative control wells resulted in no detectable spot-forming units, therefore, any well having greater than 5 spot-forming units was considered positive.

M. RNA isolation and cDNA synthesis

When available, RNA was isolated from at least 10^6 T cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Using 0.5-1 μg of RNA, cDNA synthesis was performed with the following reaction: 2 μM poly-T (Integrated DNA Technologies, Coralville, IA), 1X RT buffer (Invitrogen), 2.5 mM DTT (Invitrogen), 40 mM dNTPs (Promega, Madison, WI), 15U RNAguard (Amersham Bioscience) heated to 65 °C for 5 min and placed on ice. Superscript III reverse transcriptase (Invitrogen) was added at 100U per reaction (total volume per reaction was 20 μl) and heated to 50 °C for 50 min before it was inactivated at 85 °C for 5 min.

N. CDR3 spectratyping

The TCR V gene nomenclature used throughout this thesis was based on that reported by (Arden et al., 1995). The cDNA was amplified with 1.6 μM each of V- and C-specific primers (Han et al., 1999; Maslanka et al., 1995), 1X PCR buffer II (Applied Biosystems, Framingham, MA), 1.5 mM $MgCl_2$ (Applied Biosystems), 40 mM dNTPs (Promega), and 1U AmpliTaq DNA polymerase (Applied Biosystems) in a 25 μl volume per reaction. The cycling program was the following: 94 °C for 30 sec, 55 °C for 30 sec,

72 °C for 30 sec for a total of 30 cycles, followed by a 10 min incubation at 72 °C. PCR products (10 μl) were then boiled for 3 min with formamide dye and were subsequently put on ice. Samples were then loaded onto a 5% polyacrylamide gel and run at 2500V. Gels were read by a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA) and were analyzed using ImageQuant software (Molecular Dynamics).

O. TCR sub-cloning and sequencing CDR3 regions

Fresh PCR products (1-3 μl) from the reaction described above were ligated into the pCR4-TOPO vector (Invitrogen), which was used to transform TOP10 chemically competent cells (Invitrogen) according to the manufacturer's protocol. Individual colonies were picked for overnight 4 ml cultures. DNA was isolated with QIAprep miniprep kits (Qiagen, Valencia, CA) and the CDR3 regions were sequenced at the UMass Nucleic Acid Facility (Worcester, MA) using universal primers. Sequencing data was analyzed using EditSeq software (DNASTAR Inc., Madison, WI).

P. EBV quantification

Following the positive selection of PBMC samples for CD8+ lymphocytes, the negative fractions were used for EBV quantification. An extracellular stain for CD3-CD19+ cells gave us an estimate for the total number of B cells in each sample. Samples were digested and the DNA was isolated using a DNeasy Tissue kit (Qiagen). Each sample was normalized to contain the DNA of 10^5 total B cells. Samples were run in triplicate using the recommended protocol accompanying a LightCycler EBV quantification kit (Roche, Penzberg, Germany). An internal positive control was supplied with this kit.

CHAPTER III:

THE IDENTIFICATION OF CROSS-REACTIVE CD8 T CELL RESPONSES INVOLVING EBV-DERIVED, HLA-A2-PRESENTED, EPITOPES

Cross-reactive CD8 T cells exist with specificity for different proteins encoded by the same virus (Anderson et al., 1992; Kuwano et al., 1991), different serotypes of the same virus (Mathew et al., 1998; Mongkolsapaya et al., 2003), as well as for unrelated viruses (Brehm et al., 2002; Nilges et al., 2003; Wedemeyer et al., 2001). EBV has a large genome that encodes at least 69 proteins and innumerable T cell epitopes. Thus, we hypothesized that EBV infection induces multiple cross-reactive T cell responses, which would include T cells specific for multiple EBV proteins and memory T cells specific for a previously encountered virus. To identify cross-reactive responses, we grew CD8 T cell lines and stimulated them with more than 30 known HLA-A2-presented epitopes derived from EBV, IV, cytomegalovirus (CMV), human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV, HCV), vaccinia virus (VV), and some self-proteins, such as tyrosinase and protein phosphatase. We used the accumulation of intracellular IFNγ following short-term stimulation with any peptide other than that used to culture the T cell line as an indication of a potential cross-reactive response. This screening process revealed several different cross-reactive specificities that were present in T cell lines derived from more than one donor, all of which could potentially contribute to the immune response directed toward EBV.

A. Putative cross-reactive T cell responses involving two different EBV proteins

i. Specificity for $BMLF1_{280-288}$ and $LMP2_{329-337}$

BMLF1 (GLCTLVAML) is an early lytic viral protein, and 100% of HLA-A2+ individuals mount a CD8 T cell response against this particular epitope during the acute phase of EBV infection (Table 1) (Catalina et al., 2001). LMP2 (LLWTLVVLL) is a latent viral protein, and this particular epitope is a much less frequent target of the EBV-specific CD8 T cell response (Table 1) (Catalina et al., 2001). BMLF1-specific T cell lines derived from two healthy donors produced IFNγ in response to stimulation with LMP2 peptide (20-26%), but not in response to tyrosinase or other non-specific stimulations (0.2-1%) (Figure 5A, C). Control T cell lines derived from the same two donors produced little to no IFNγ following LMP2 stimulation, further suggesting that the T cell population responding to LMP2 was growing in response to BMLF1-pulsed T2 cells and not in response to T2 cells alone (Figure 6A, B). We later confirmed this cross-reactive response by using a similar BMLF1-specific T cell line derived from donor D-002 to assess the ability of these cross-reactive cells to simultaneously bind BMLF1 tetramer while producing IFNγ to LMP2 stimulation. In this case, the majority of the IFNγ production to LMP2 was coming from the BMLF1 tetramer-positive population (Figure 5B). These two epitopes are similar in sequence, sharing 5 of 9 aa residues. Thus, molecular mimicry may be the mechanism behind this cross-reactive interaction since three of the shared residues are at consecutive central positions (P4-6) along the epitope sequence and are likely available for TCR engagement. However, it is interesting to note that we were unable to cultivate an LMP2-specific T cell line from donor D-002.

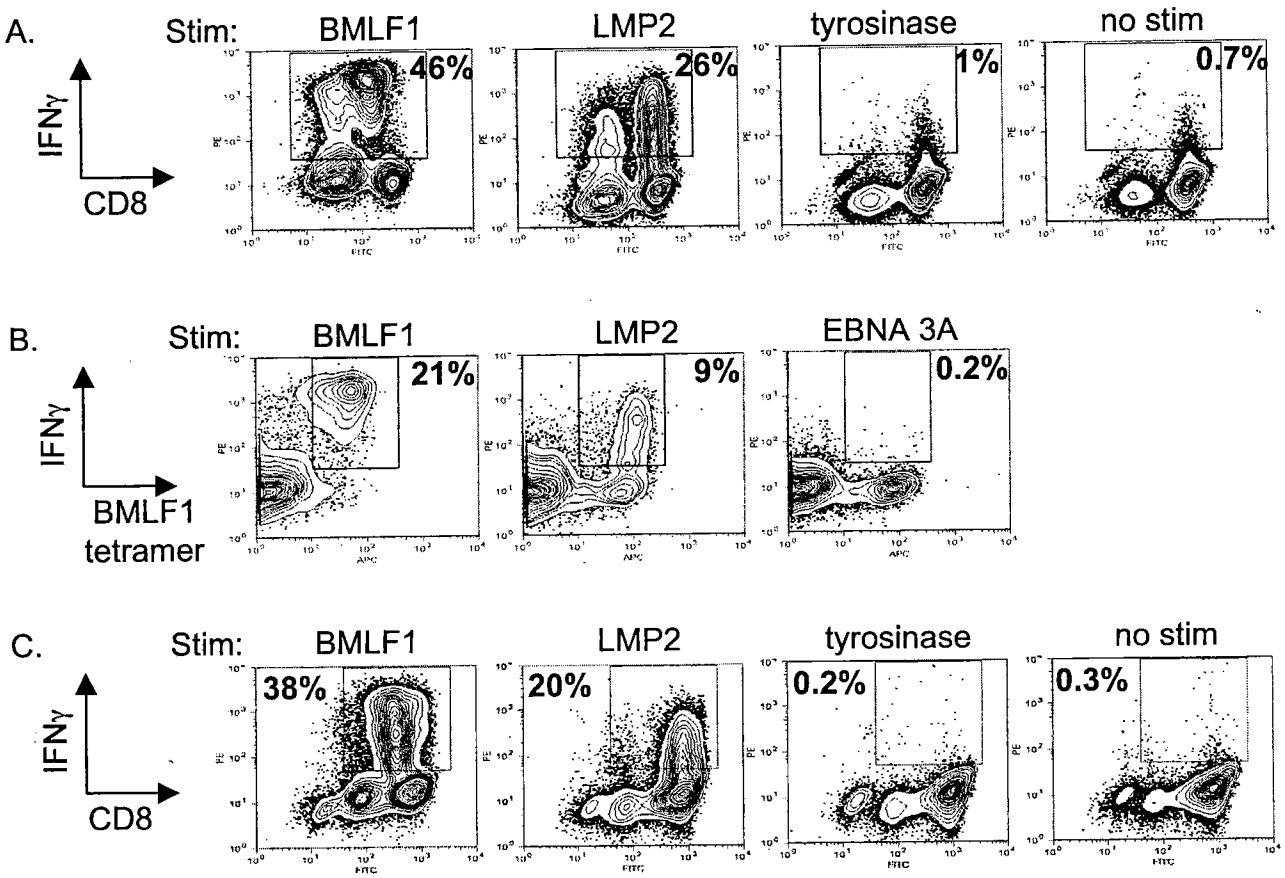


Figure 5. Cross-reactive T cells with specificity for EBV-BMLF1 and EBV-LMP2. T cell lines were cultured for 4-8 weeks with BMLF1 peptide-pulsed T2 cells. Cells were derived from healthy donors (A, B) D-002 and (C) D-012. (A, C) Standard intracellular IFNγ assays are shown, where T cell lines were stimulated with EBV-LMP2 or a non-specific peptide, tyrosinase. Positive and negative controls are also provided, BMLF1 or no stimulation respectively. Percentage of T cell line producing IFNγ is shown. (B) Intracellular IFNγ stain combined with an extracellular BMLF1 tetramer stain, where EBV-EBNA 3A served as a non-specific stimulation control. Percentage of T cell line binding BMLF1 tetramer and producing IFNγ is shown.

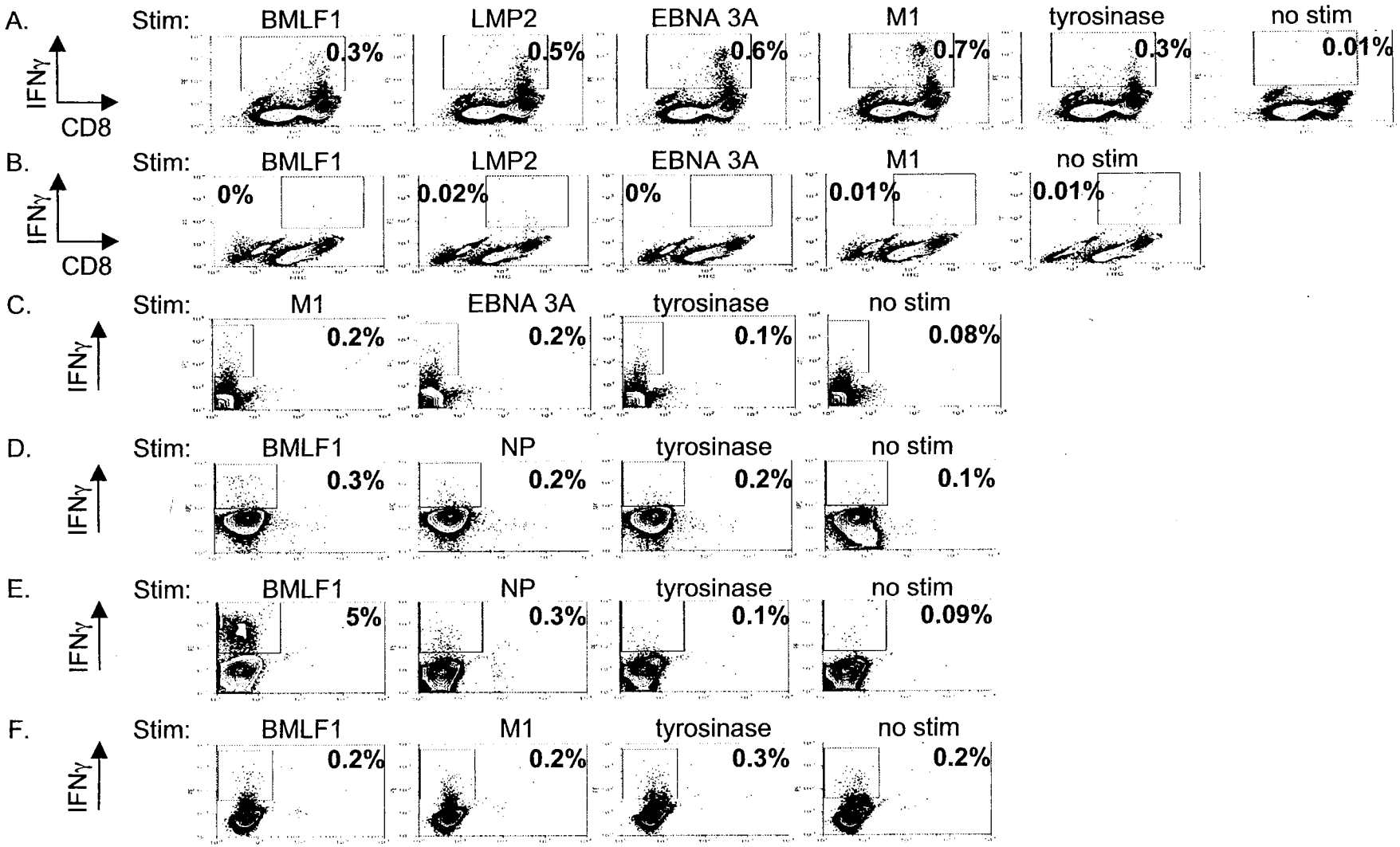


Figure 6. Control T cell lines lack specificity for HLA-A2-presented peptides. T cell lines were cultured for 3-8 weeks in the presence of unpulsed T2 cells. Standard intracellular IFNγ stains are shown, where the peptide used for stimulation is shown above each plot and the percentage corresponds to the fraction of the T cell line producing IFNγ. Cells were derived from healthy donors (A) D-002, (B) D-012, (C) D-046, (F) D-042 and IM patients (D) E1101, (E) E1109. (C-F) Plots show the FL1 channel on the x-axis, but no antibody in the assay was detectable in that channel.

After 8 weeks of culturing, only 4% of the CD8 T cells produced IFNγ in response to LMP2 and this line did not respond at all to BMLF1 stimulation, exemplifying the non-reciprocal nature of CD8 T cell cross-reactivity (data not shown). Furthermore, BMLF1-specific lines derived from other unrelated donors did not share this particular pattern of cross-reactivity. T cell lines derived from only 2 out of 6 healthy immune donors and 0 out of 5 IM patients displayed evidence of a cross-reactive response involving the EBV-derived BMLF1 and LMP2 epitopes.

ii. Specificity for $BMLF1_{280-288}$ and $BRLF1_{109-117}$

Like BMLF1 (GLCTLVAML), BRLF1 (YVLDHLIVV) is a lytic viral protein, but with an immediate early expression profile. Both epitopes are frequent targets of the primary and memory EBV-specific CD8 T cell responses (Hislop et al., 2002) (Table 1). These two epitopes have no sequence similarity, yet data we derived from three different individuals suggested that T cells existed that could recognize both epitopes. The most convincing evidence of this cross-reactive interaction was the ex vivo detection of CD8 T cells that co-stained with both BMLF1- and BRLF1-loaded tetramers, corresponding to 0.3% of the CD8 T cell population found in the peripheral blood of a patient, E1232, undergoing a primary immune response to EBV and presenting with symptoms of infectious mononucleosis (IM) (Figure 7A). Such a staining profile was not evident when this T cell population co-incubated with BMLF1- and (IV)M1-loaded tetramers, suggesting that the interaction was indeed antigen-specific. Cross-reactive T cells that can co-stain with two distinct tetramers likely reflect a high avidity interaction with both epitopes. However, we have also observed cases where cross-reactive T cells presumably

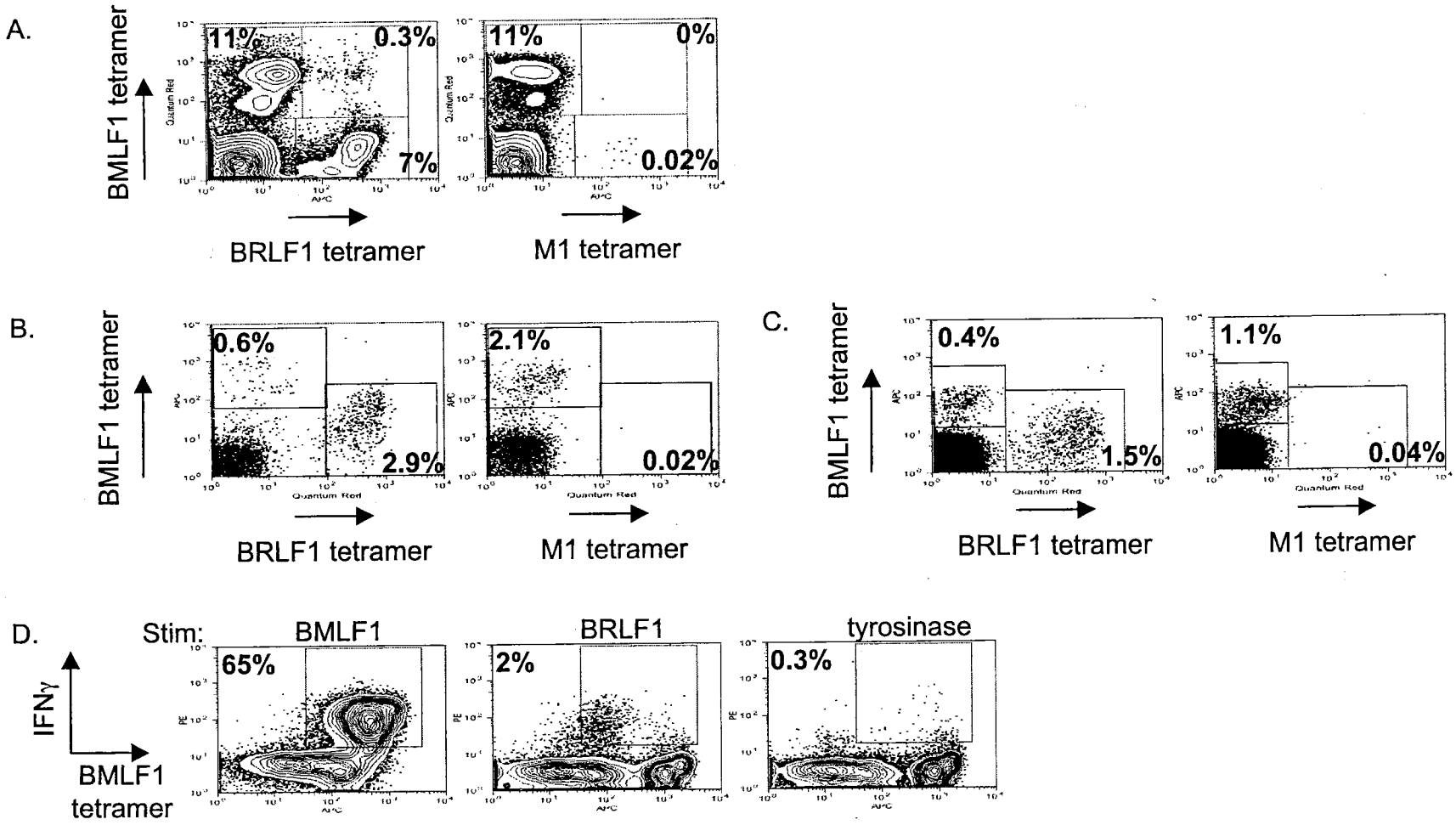


Figure 7. Cross-reactive T cells with specificity for EBV-BMLF1 and EBV-BRLF1. (A-C) CD8 T cells freshly isolated from IM patients (A) E1232 at day 13 post-presentation and (B, C) E1205 at days 0 and 19 post-presentation were stained extracellularly with tetramers, where M1-loaded tetramers served as the negative control. (D) A T cell line derived from healthy donor D-002 was cultured with both BMLF1 and BRLF1 peptide-pulsed T2 cells simultaneously for 4 weeks. A standard intracellular IFNγ assay was combined with an extracellular BMLF1 tetramer stain, and the percentage of the T cell line that binds tetramer and produces IFNγ is shown.

had a higher avidity for one epitope over the other. For example, co-incubating freshly isolated CD8 T cells derived from IM patient E1205 with BMLF1- and BRLF1-loaded tetramers did not result in double tetramer-positive cells. Rather, in the presence of BRLF1-loaded tetramer, the percentage of BMLF1 tetramer-positive cells declined to 0.6% compared to a frequency of 2.1% when in the presence of a non-specific (IV)M1-loaded tetramer (Figure 7B). The same trend was observed later during the acute phase of EBV infection, when the frequency of BMLF1 tetramer-positive cells declined to 0.4% in the presence of BRLF1-loaded tetramer as opposed to a starting frequency of 1.1% in the presence of the non-specific M1-loaded tetramer (Figure 7C). This data suggested that this population of cross-reactive T cells had a higher avidity for the BRLF1 epitope than the BMLF1 epitope so that under competitive conditions, these cells would preferentially bind BRLF1-loaded tetramer. Thus, this particular pattern of cross-reactivity can be subtle but may frequently occur during the acute phase of EBV infection since ex vivo tetramer staining indicated that 2 out of 4 IM patients mounted this cross-reactive T cell response.

In an attempt to culture cross-reactive cells specific for BMLF1 and BRLF1, we grew T cells isolated from healthy donor D-002 in the presence of a 1:1 mixture of BMLF1- and BRLF1-pulsed T2 cells. These conditions supported the growth of cross-reactive cells that dimly stained BMLF1 tetramer, once again suggestive of a lower avidity interaction with BMLF1, while producing IFNγ specifically to BRLF1 stimulation (Figure 7D). We were unable to detect this cross-reactive T cell response in 4 other healthy donors but, in those cases, only BMLF1-pulsed T2 cells were used for T cell

culturing and that, presumably low avidity, TCR stimulation may not have been sufficient to support the growth of this specific cross-reactive T cell population.

B. Putative cross-reactive T cell responses involving two unrelated viruses, EBV and IV

i. Specificity for $BMLF1_{280-288}$ and $M1_{58-66}$

The immunodominant M1 epitope (GILGFVFTL) is a target of the IV-specific CD8 T cell response within most HLA-A2+ individuals and shares only 3 of 9 aa residues with the immunodominant BMLF1 epitope (GLCTLVAML) derived from EBV (Tables 1, 3). Using both BMLF1- and M1-specific T cell lines, we have been able to demonstrate the production of IFNγ in response to stimulation with the opposite peptide. As examples, 6% of a BMLF1-specific T cell line derived from healthy donor D-042 produced IFNγ following M1 stimulation, while 3% of an M1-specific T cell line derived from healthy donor D-002 produced IFNγ following BMLF1 stimulation (Figure 8A, B). Control T cell lines derived from each donor and grown in the presence of T2 cells alone did not produce IFNγ following M1 (0.2%) or BMLF1 (0.3%) stimulation respectively (Figure 6 F, A). Furthermore, using T cells isolated from donor D-042, we cultivated a second EBV-specific line using T2 cells pulsed with an $LMP2_{426}$ peptide (66% of the line was specific based on IFNγ production) and showed that the cross-reactive M1-specific response was not present (0.1% produced IFNγ to M1) (data not shown). Therefore, we deduced that M1-specific T cells grew specifically to the BMLF1-pulsed T2 cells (Figure 8A).

We demonstrated the simultaneous recognition of (EBV) BMLF1 and (IV) M1

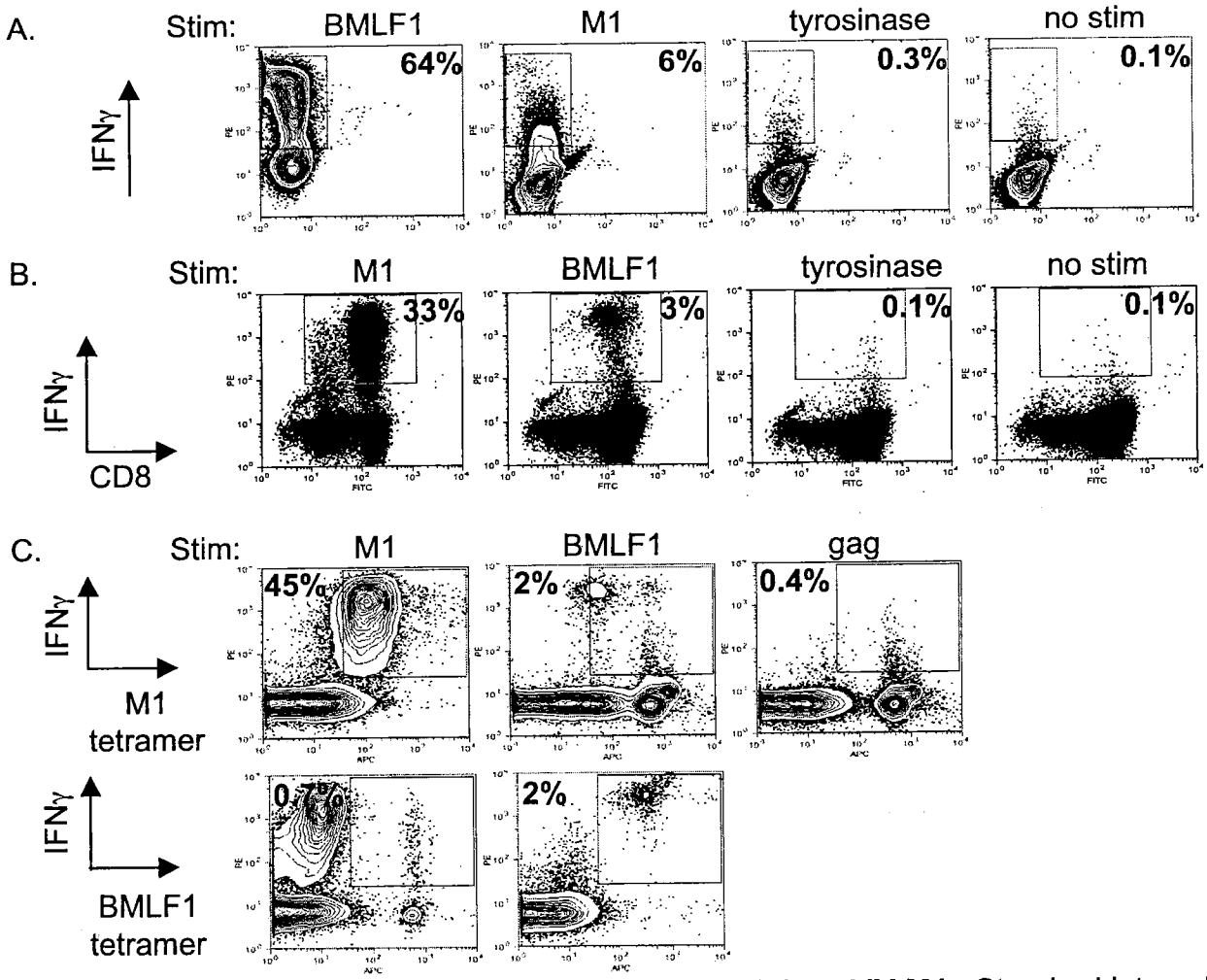


Figure 8. Cross-reactive T cells with specificity for EBV-BMLF1 and IV-M1. Standard intracellular IFNγ assays on T cell lines cultured for 3-5 weeks with (A) BMLF1 peptide-pulsed T2 cells or with (B, C) M1 peptide-pulsed T2 cells. Cells were derived from healthy donors (A) D-042 and (B) D-002 or from (C) IM patient E1101. The percentage of the T cell line that produces IFNγ is shown, except in (C) where the percentage reflects the proportion of the T cell line that produces IFNγ and binds tetramer. An HIV-derived gag peptide served as the non-specific stimulation control in that experiment. (A) Plots show the FL1 channel on the x-axis, but no antibody in the assay was detectable in that channel.

using a combination tetramer and intracellular IFNγ stain. Within an M1-specific T cell line derived from IM patient E1101, 2% of the cells produced IFNγ following BMLF1 stimulation while simultaneously binding M1 tetramer with a range of intensities (Figure 8C). In the opposite stain, the same T cell line contained a smaller population of cells (0.7%) that bound BMLF1 tetramer while simultaneously producing IFNγ to M1 stimulation (Figure 8C). Overall, we were able to detect T cells with this cross-reactive specificity within T cell lines derived from 3 out of 8 healthy immune donors. We were also able to detect these cross-reactive T cells in the blood of 2 out of 8 IM patients, which will be further discussed in Chapter IV of this thesis.

ii. Specificity for $BMLF1_{280-288}$ and NP_{85-94}

NP (KLGEFYNQMM) is a subdominant IV-derived epitope among HLA-A2+ individuals, with an aa sequence dissimilar from that of the immundominant EBV-derived BMLF1 epitope (GLCTLVAML) (Tables 1, 3). BMLF1-specific cell lines derived from two different IM patients had a small population of T cells (2%) that produced IFNγ in response to NP stimulation (Figure 9A, B). Control T cell lines derived from the same two donors did not contain this subset of NP-responsive cells (0.2-0.3%), suggesting that their growth was due to the presence of BMLF1 peptide in the culture (Figure 6D, E). In patient E1109, we were able to confirm that this 2% subset of NP-responsive cells was able to bind BMLF1 tetramer, providing more definitive evidence that these T cells could engage both of these structurally dissimilar epitopes (Figure 9C).

It is interesting that we were only able to culture T cells with this cross-reactive specificity from the blood of IM patients (2 out of 4) and not from the blood of 7 different

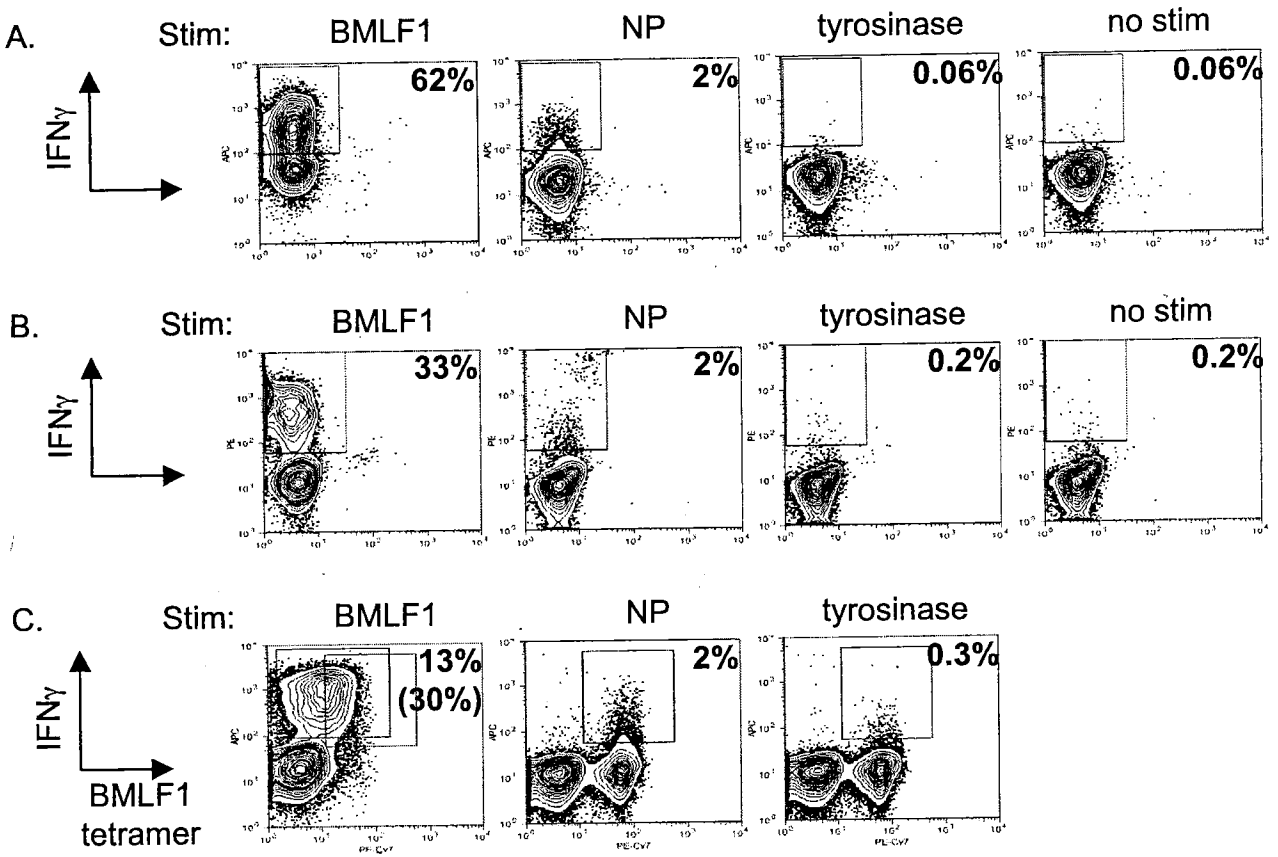


Figure 9. Cross-reactive T cells with specificity for EBV-BMLF1 and IV-NP. T cell lines were cultured for 4-5 weeks with BMLF1 peptide-pulsed T2 cells. Cells were derived from IM patients (A) E1101 and (B, C) E1109. (A, B) Standard intracellular IFNγ assays are shown, where the percentage represents the proportion of the T cell line producing IFNγ. (C) Intracellular IFNγ stain combined with an extracellular BMLF1 tetramer stain, where tyrosinase served as a non-specific stimulation control. Percentage of T cell line binding BMLF1 tetramer and producing IFNγ is shown. Total IFNγ production in response to BMLF1 is shown in parenthesis, as TCR downregulation may have prevented efficient co-staining with the BMLF1 tetramer.

healthy immune donors. As mentioned, the NP-specific T cell response is thought to be subdominant. Since very little is known about the primary T cell response to a natural human IV infection, the definition of subdominant is based mostly on the fact that it has been difficult to detect NP-specific memory T cells in healthy immune donors. The fact that we were only able to detect these otherwise low frequency T cells in IM patients may indicate that some NP-specific memory T cells have proliferated and increased in number in vivo as a result of cross-reactive stimulation with BMLF1 and possibly other EBV-derived epitopes. This hypothesis will require further investigation.

iii. Specificity for EBNA $3A_{596-604}$ and $M1_{58-66}$

We also have preliminary evidence to suggest cross-reactive T cell specificity for an EBV epitope other than BMLF1. EBNA 3A is a latent EBV protein and a frequent target of the CD8 T cell response, particularly during the persistent phase of EBV infection (Catalina et al., 2001). The EBNA 3A epitope (SVRDRLARL) shares only 1 of 9 aa residues with the immunodominant IV-derived M1 epitope (GILGFVFTL) (Tables 1, 3), however, the M1-specific T cell lines derived from three separate healthy donors all produced IFNγ in response to EBNA 3A stimulation (Figure 10). This cross-reactive T cell subset sometimes represented only a small proportion of the T cell line (D-012, 4% and D-046, 2%) but sometimes included over half of the T cell population within that line (D-002, 56%). Such an extensive population of EBNA 3A-responsive cells was not observed in the control T cell line derived from D-002 (0.6%), and was absent from the control T cell lines derived from donors D-012 and D-046 (Figure 6A-C).

A cross-reactive response involving EBNA 3A would result in a second

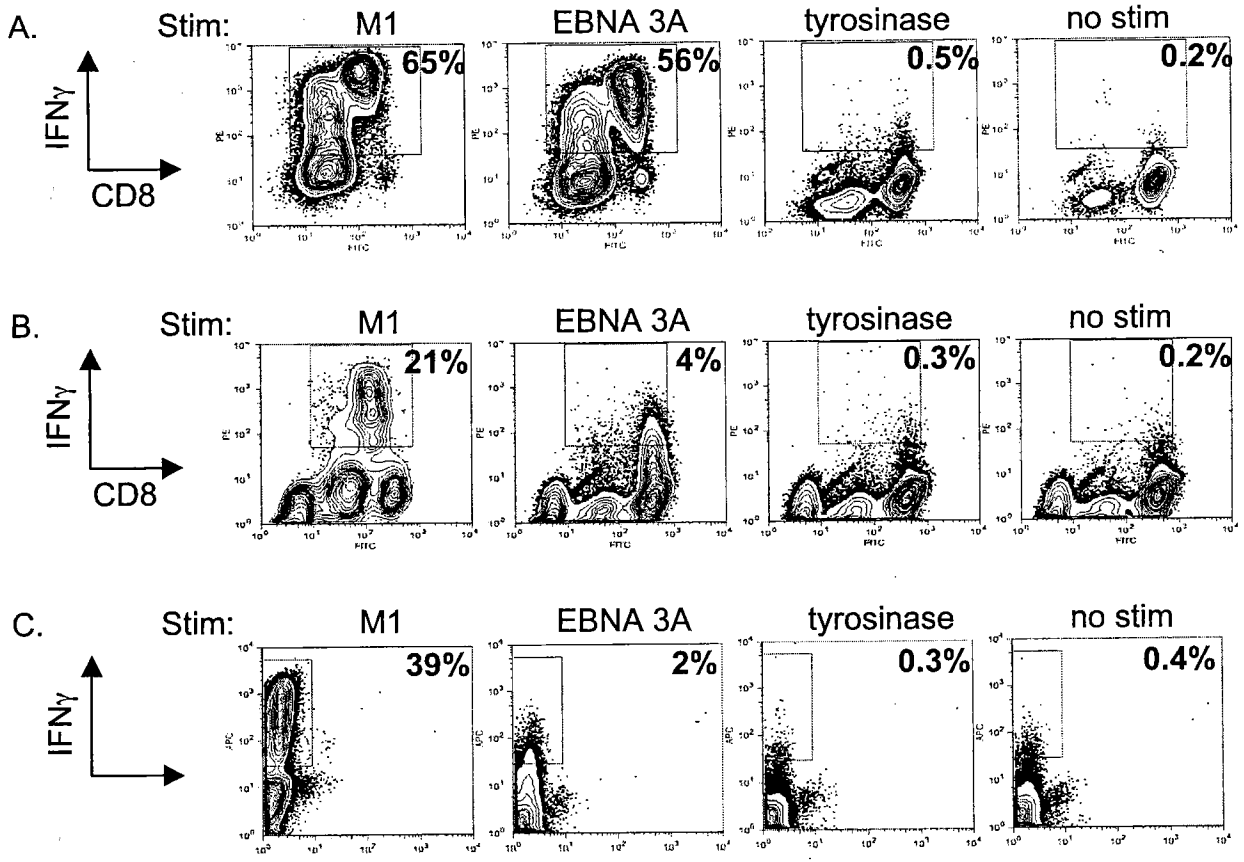


Figure 10. Cross-reactive T cells with specificity for EBV-EBNA 3A and IV-M1. T cell lines were cultured for 3-8 weeks in the presence of M1 peptide-pulsed T2 cells. Standard intracellular IFNγ stains are shown, where the peptide used for stimulation is shown above each plot and the percentage corresponds to the fraction of the T cell line producing IFNγ. Cells were derived from healthy donors (A) D-002, (B) D-012, (C) D-046.

opportunity to reactivate M1-specific memory cells and would do so during a different phase of the viral lifecycle, as BMLF1 is expressed during the lytic phase and EBNA 3A is expressed during the latent phase. We detected the cross-reactive T cell response specific for EBNA 3A and M1 in T cell lines derived from 3 out of 8 healthy immune donors. This was the same frequency with which we detected the cross-reactive T cell response specific for BMLF1 and M1, and 2 of the 3 responding donors displayed evidence of having both cross-reactive patterns. However, this cross-reactive response has not yet been detected in T cell lines derived from IM patients.

C. The individual variation in cross-reactive specificities and the non-reciprocal pattern of cross-reactive T cell responses

All of the EBV seropositive HLA-A2+ individuals enrolled in our study have had a BMLF1-specific T cell population, but the presence of a cross-reactive subset within that population, as well as the alternative specificity of those cross-reactive cells, varied between individuals. This observation may reflect the clonal diversity of the BMLF1-specific population and, therefore, the private specificity of each individual's TCR repertoire. Furthermore, any factors that affect antigen presentation will inevitably influence the activation and magnitude of a cross-reactive T cell response. The expression pattern of viral proteins and epitope processing affect the time, location, and concentration of antigen presented to T cells. If the presentation of an alternative epitope is less than optimal, it may prevent the development of a cross-reactive response in vivo. Consequently, any cross-reactive T cell clones present at relatively low frequency in vivo can be easily diluted out in bulk culture by the growth of more frequent, perhaps non-

crossreactive, T cell clones.

Through the study of cultured T cell lines, we have also observed non-reciprocal or unequal patterns of cross-reactive responses. In the case of cross-reactive responses involving BMLF1 and M1, we often found that the frequency of cells producing IFNγ in response to a cross-reactive stimulation was higher in M1-specific T cell lines (3%) than in BMLF1-specific T cell lines (1%) (Figure 11A, B). The difference between T cell lines in the detection of cross-reactive T cells was even more pronounced in a separate experiment when we assessed another effector function, MIP-1β production. In this case, the frequency of cross-reactive cells in the M1-specific T cell line increased to at least 16%, but cross-reactive cells were undetectable in the BMLF1-specific T cell line using this assay (Figure 11A, B). While it is still unclear if these unequal patterns of cross-reactivity will hold true with T cells having other specificities, the non-reciprocal nature of T cell cross-reactivity has previously been observed. Using murine models of heterologous virus infection, the sequence of infections was determined to influence protective immunity (Selin et al., 1998). For instance, LCMV-immune mice were protected against a lethal VV infection, and subsets of LCMV-specific memory T cells proliferated (ie. increased in number) following stimulation with VV; however, VV-immune mice were not protected from LCMV infection, and VV-specific memory T cells did not proliferate following stimulation with LCMV (Kim et al., 2002). Since that observation, it has also become apparent that cross-reactive T cell clones, based on cytokine production, proliferate with different efficiencies in vivo and that this may be attributed to differences in TCR avidity for the alternative epitope

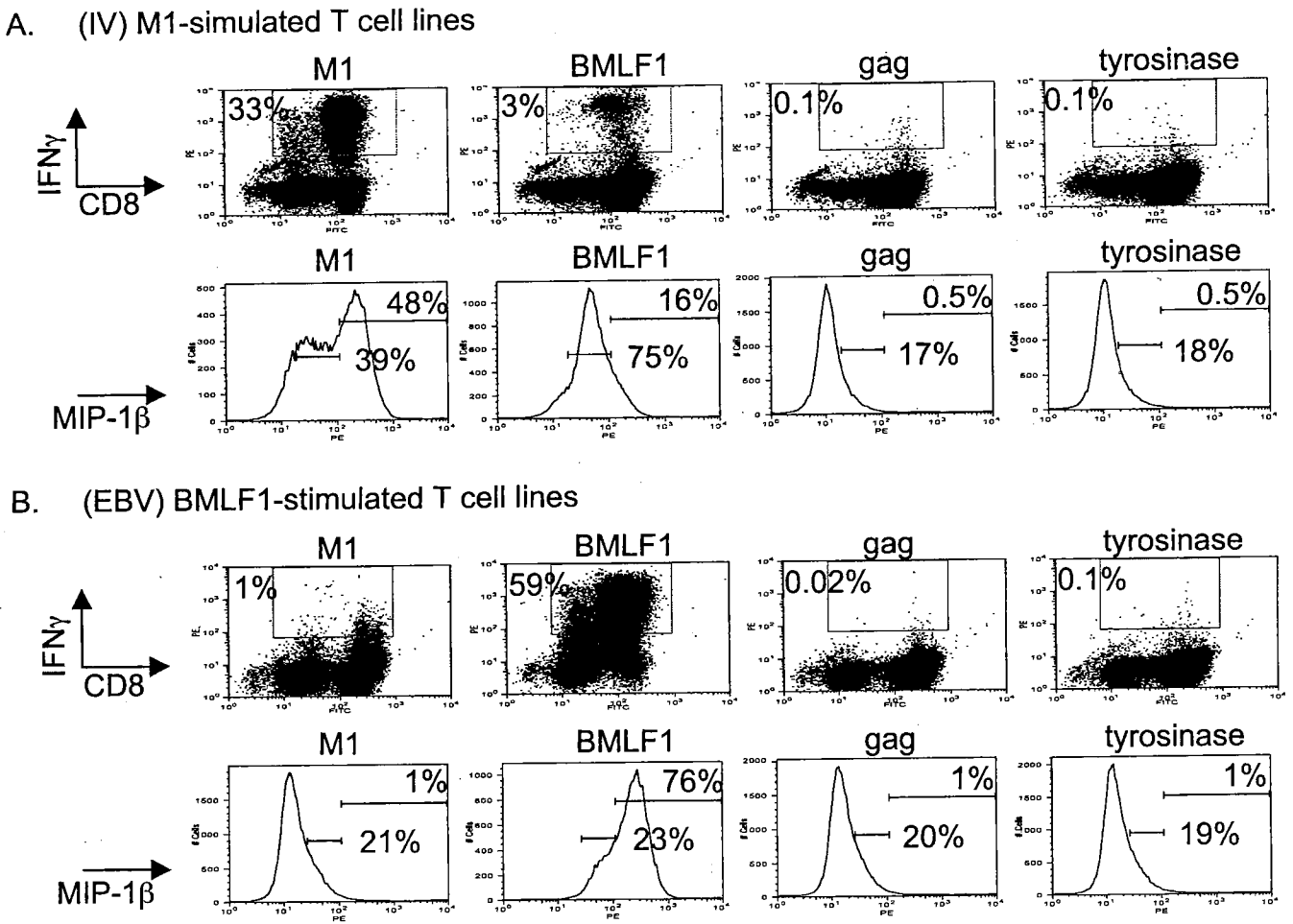


Figure 11. Unequal patterns of cross-reactivity among T cell lines. T cell lines derived from healthy donor D-002 were cultured for 3-4 weeks in the presence of (A) M1 or (B) BMLF1 peptide-pulsed T2 cells. Standard intracellular cytokine stains are shown, where the peptide used for stimulation is shown above each plot and the percentage corresponds to the fraction of the T cell line producing cytokine.

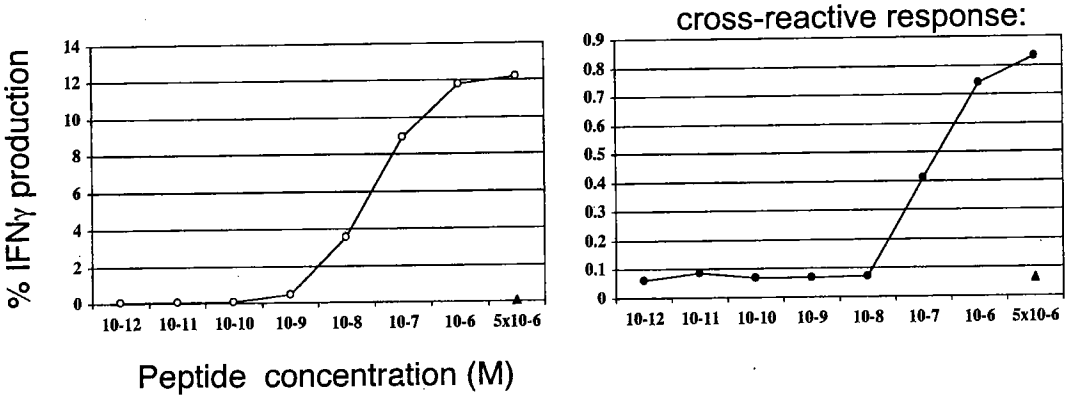
(Cornberg et al., 2005b). Thus, the stimulating antigen used to culture cross-reactive T cell populations may only promote the outgrowth of a select subset of cross-reactive clones having high avidity for that antigen.

D. Avidity of the cross-reactive TCR interaction with alternative epitopes

As mentioned above, a difference in avidity for the two epitopes may explain the unequal patterns of cross-reactive T cell responses we have observed in cultured T cell lines. To assess and compare the avidity of the interaction between cross-reactive T cells and the M1 or the BMLF1 epitope, we used a decreasing titration of peptide in a standard intracellular IFNγ assay. The cross-reactive population within the M1-specific T cell line, derived from healthy donor D-002, had a relatively similar avidity for M1 and BMLF1 peptide, reaching half the maximum IFNγ production between 10^{-7} M (BMLF1) and $5x10^{-8}$ M (M1) (Figure 12A). In contrast, the cross-reactive population within the BMLF1-specific T cell line, derived from the same donor, had at least a 100-fold higher avidity for the M1 peptide, the alternative ligand, over the BMLF1 peptide, reaching half the maximum IFNγ production at a 10^{-9} M concentration of M1 and at a $5x10^{-7}$ M concentration of BMLF1 (Figure 12B). These data imply that culturing CD8 T cells with BMLF1 peptide selects for a smaller population of cross-reactive cells with very high avidity for the alternative epitope, M1, while culturing CD8 T cells with M1 peptide promotes the outgrowth of a larger population of cross-reactive cells with an equal or slightly lower avidity for the alternative epitope, BMLF1. The reason for this may stem from the structure of each epitope and its influence on TCR repertoire development.

The M1 epitope is presented to the TCR in a rather featureless orientation that

A. (IV) M1-simulated T cell line



B. (EBV) BMLF1-stimulated T cell line

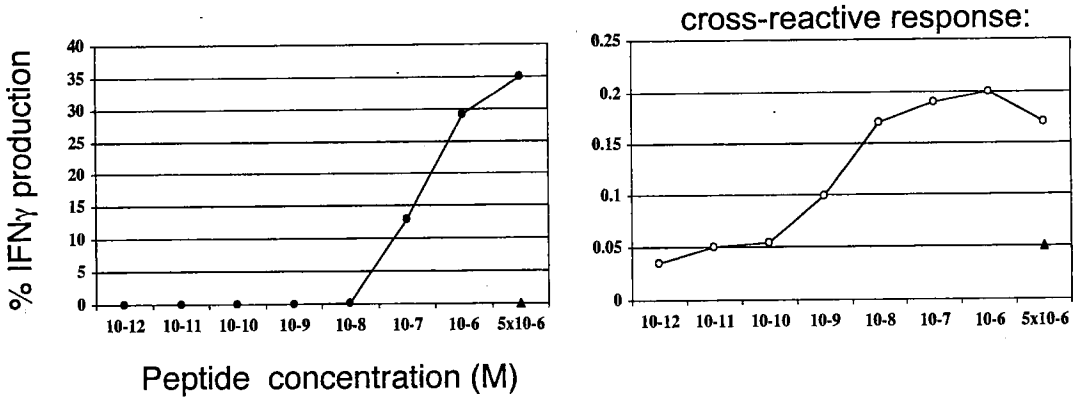


Figure 12. Cross-reactive T cells interact with each epitope with different avidities. T cell lines, derived from healthy donor D-002, had been cultured for 4 weeks in the presence of (A) M1 or (B) BMLF1 peptide-pulsed T2 cells and were used in a standard intracellular IFNγ assay that included a titration of peptide concentrations. (▲ tyrosinase, O M1, ● BMLF1 stimulation)

likely imposes strict requirements on the structure of the TCR with which it interacts, namely Vβ17/Jβ2.7/CDR3β *RS*, and it is reasonable to assume then that the interaction is of relatively high avidity (Stewart-Jones et al., 2003). If that strict M1-specific TCR structure, which comprises the bulk of the M1-specific repertoire, is also capable of interacting with BMLF1, then it is not surprising that the majority of an M1-specific T cell line responds to BMLF1 at some level, such as illustrated in Figure 11A. In fact, data presented in Chapter V of this thesis will reveal that BMLF1-specific T cells can express Vβ17, the dominant Vβ17+ clone found in healthy donor D-002 expressing Vβ17/Jβ2.7/CDR3β IDGTA.

In contrast to the M1 epitope, the BMLF1 epitope does not appear to impose such strict TCR requirements, as will be discussed in Chapter V of this thesis. The BMLF1-specific repertoire includes a more diverse array of TCR structures that likely have a diverse array of avidities for the BMLF1 epitope, and those T cells expressing Vβ17 represent only a small proportion. Thus, it is likely that only a small proportion of the BMLF1-specific repertoire will meet the strict requirements necessary for forming a stable interaction with the M1 epitope. It is not completely clear why the small population of T cells within a BMLF1-specific T cell line interact with the M1 epitope with higher avidity than the M1-specific T cell line, but perhaps the comparison was not valid. The M1-specific T cell line is highly polyclonal and the relative avidity measurement we observed represents the average avidity of all the M1-specific clones within that line. In contrast, the cross-reactive M1-specific population within the BMLF1-specific T cell line is likely to be oligoclonal because, despite coming from a

diverse array of BMLF1-specific TCR structures, most Vβ families within this line are comprised of only 1 or 2 unique clones. Thus, the ideal experiment comparing the avidities of the non-crossreactive and cross-reactive TCR interaction with the M1 epitope will require individual T cell clones.

E. Chapter III Summary

We predicted that the large EBV genome encodes many T cell epitopes that can simultaneously activate primary T cells and pre-existing cross-reactive memory T cells in an antigen-specific manner. In this study alone, we detected cross-reactive T cell responses with five different specificities that involved EBV-derived antigens. Each of these cross-reactive responses has the potential to participate in the collective immune response to acute EBV infection. Part of our hypothesis was that the contribution of these cross-reactive T cell responses to the immune response promoted the development of immune-mediated pathology, such as infectious mononucleosis, because the symptoms and clinical signs of IM are thought to be caused by the tissue-infiltrating, EBV-specific, T cells and the cytokines they secrete (Rickinson and Kieff, 2001). We next aimed to determine if these cross-reactive T cell responses contributed to the magnitude and quality of the total EBV-specific T cell response and, therefore, to the severity of infectious mononucleosis.

There is no dispute that the in vivo environment during an acute viral infection cannot be mimicked in vitro. In order to determine the physiological relevance of the cross-reactive responses we detected in T cell cultures, we next aimed to detect these cells ex vivo, in blood samples donated by IM patients. However, these in vitro studies

have revealed certain features of T cell cross-reactivity that one must consider when attempting to detect cross-reactive T cells:

1) At least 4 of the 5 cross-reactive specificities reported here involved epitopes with little to no sequence similarity. Molecular mimicry is just one form of T cell cross-reactivity and, therefore, assays developed for screening cross-reactive responses should not be limited to peptides having similar sequence.
2) When detecting cross-reactive T cell responses, multiple different assays must be employed. While co-staining with two tetramers can result in a double-positive cell population, cross-reactive cells may have different avidities for each epitope and, under competitive conditions, may preferentially bind one tetramer over the other. Functional assays can also be deceiving. We reported here that, within an M1-specific T cell line, a higher frequency of T cells produced MIP-1β than IFNγ following cross-reactive stimulation with BMLF1 peptide.
3) Despite having the same acute viral infection, and even having T cell memory to the same previously encountered virus, the frequency and specificity of cross-reactive T cell responses remains unique to each individual. Based on the literature, this observation may be attributed to each individual's unique history of infections and their private repertoire of T cell clones.

CHAPTER IV:

CROSS-REACTIVE CD8 T CELLS SPECIFIC FOR EBV-BMLF1$_{280-288}$ AND IV-M1$_{58-66}$ CONTRIBUTE TO THE LYMPHOPROLIFERATION IN EBV-ASSOCIATED INFECTIOUS MONONUCLEOSIS

There is a high degree of individual variation in disease severity associated with human virus infections, and age is one of many factors that can contribute to such variation. Childhood infections with EBV are often sub-clinical, while the same infection is frequently symptomatic in adolescents and adults and presents as infectious mononucleosis. IM can vary in duration, from a few weeks to 6 months, and the symptoms can vary in severity (Rea et al., 2001). Complications, such as pneumonia and fulminant hepatitis, are more common in older adults and have been linked to the infiltration of activated T cells and EBV-infected B cells into these tissues (Auwaerter, 1999; Axelrod and Finestone, 1990; Rickinson and Kieff, 2001). When comparing IM and asymptomatic cases of acute EBV infection, Silins et al. found that the magnitude of the CD8 T cell response, not viral load, correlated with the presence or absence of disease (Silins et al., 2001). Furthermore, treatment of IM patients with anti-viral drugs, although decreasing viral load, did not have any effect on the disease course (Andersson et al., 1987; Torre and Tambini, 1999). These data suggest that a massive CD8 T cell response can be counterproductive and mediate the disease pathology. It is still unclear why this massive CD8 T cell proliferation occurs more frequently in older individuals.

Based on murine models of heterologous immunity which showed that T cells specific to a previously encountered virus may enhance the immunopathology during a

second unrelated virus infection, and based on the increasing number of reports documenting CD8 T cell cross-reactivity between viruses, we hypothesized that cross-reactive memory T cells specific to previously encountered pathogens contributed to this massive CD8 T cell proliferation during acute EBV infection (Brehm et al., 2002; Mongkolsapaya et al., 2003; Nilges et al., 2003; Selin et al., 1994; Wedemeyer et al., 2001; Welsh et al., 2004). In support of this, there is well-documented evidence that at least a proportion of the CD8 T cells activated by EBV can have alternative specificities for allogeneic MHC molecules, self-peptides, and bacterial antigens (Burrows et al., 1994; Misko et al., 1999; Strang and Rickinson, 1987; Tomkinson et al., 1989). Furthermore, as presented in Chapter III of this thesis, our preliminary data revealed at least five different cross-reactive T cell specificities with the potential to contribute to an immune response to EBV. Here we present the results of a more thorough investigation of one of those cross-reactive T cell specificities, that involving EBV-BMLF1 and IV-M1. We were able to further characterize this cross-reactive response in vitro using T cell clones and multiple functional assays, and demonstrated the physiological relevance of this response through the detection of these cross-reactive T cells in the blood of 2 out of 8 IM patients, both of which had notably skewed M1-specific memory TCR repertoires that were suggestive of cross-reactive T cell expansions. These data provide more support for our hypothesis that cross-reactive T cell responses contribute to the development and severity of EBV-associated infectious mononucleosis.

A. Maintenance of cross-reactive T cells in the memory pools of healthy donors

Using the previously mentioned intracellular IFNγ assay to screen T cell lines for

cross-reactive responses, we observed that 3 out of 8 healthy donors (38%) with previous exposure to EBV and IV had cross-reactive T cells with specificity for EBV-BMLF1 and IV-M1 within their memory CD8 T cell population (Table 4). In an attempt to determine the frequency of these cross-reactive T cells in the peripheral blood, we co-stained CD8 T cells ex vivo with BMLF1- and M1-loaded tetramers. A population of double tetramer-postive cells (0.01%) was detectable when 10^6 events were collected, confirming our ability to culture them in vitro (Figure 13A). This frequency may be an underestimate given the potential for cross-reactive T cells to have a higher avidity for one epitope over the other (Figure 7). Using this technique, we also demonstrated that the resting state frequency of BMLF1- and M1-specific memory T cell populations, which would include any cells with cross-reactive potential, were relatively stable over time (Figure 13B).

B. Breadth and quality of this cross-reactive T cell response

Further investigation into the effector function of these cross-reactive T cells required a larger number of cells than could be isolated ex vivo. We varied the stimulation protocol to optimize the growth of cross-reactive T cells. We tried the following three techniques: 1) culture T cells with M1 peptide during week 1, BMLF1 peptide during week 2, and M1 peptide during week 3, 2) culture T cells with BMLF1 peptide during week 1, M1 peptide during week 2, and BMLF1 peptide during week 3, 3) culture T cells with M1 and BMLF1 peptides simultaneously for all 3 weeks. Based on this preliminary experiment, we determined that the growth of cross-reactive T cells was optimal following the simultaneous stimulation with a 1:1 ratio of BMLF1- and M1-pulsed T2 cells (Figure 14A). The frequency of cells that co-stained with both BMLF1-

Table 4. CD8 T cell lines from multiple healthy donors responding to M1 and BMLF1 stimulation

(IV) M1-stimulated T cell lines:

Donor	PMA+Ionomycin	(IV)M1	(EBV)BMLF1	(HIV)gag	Tyrosinase	No peptide
D-002	56.9	33.2	**2.8**	0.1	0.1	0.1
D-012	78.9	23.5	**1.1**	**	0.5	0.1
D-042	94.9	17.1	**1.2**	0.5	0.6	0.4
D-035	45.0	11.9	0.6	**	0.6	0.5
D-044	70.2	2.4	1.9	0.2	0.2	0.1
D-045	8.6	9.2	1.3	**	1.7	0.8
D-046	72.4	38.0	0.2	0.1	0.1	0.2
D-048	16.1	7.5	0.3	0.6	0.5	0.2

(EBV) BMLF1-stimulated T cell lines:

Donor	PMA+Ionomycin	(IV)M1	(EBV)BMLF1	(HIV)gag	Tyrosinase	No peptide
D-002	76.3	**1.0**	59.0	0.0	0.1	0.0
D-012	76.9	**0.4**	35.7	**	0.1	0.1
D-042	70.4	**5.5**	63.8	0.2	0.3	0.1
D-035	18.5	0.6	20.2	**	0.3	0.2
D-044	93.8	0.6	67.6	0.2	0.6	0.5
D-045	***	***	***	***	***	***
D-046	81.1	0.2	38.5	0.1	0.1	0.1
D-048	27.0	0.4	14.0	0.5	0.5	0.4

Control T cell lines grown with unpulsed T2 cells:

Donor	PMA+Ionomycin	(IV)M1	(EBV)BMLF1	(HIV)gag	Tyrosinase	No peptide
D-002	50.2	0.1	0.8	0.1	0.1	0.0
D-012	94.9	0.0	0.0	**	**	0.0
D-042	95.9	0.2	0.2	0.3	0.3	0.2
D-035	23.6	1.6	0.2	**	0.2	0.2
D-044	64.5	0.3	1.9	0.1	0.1	0.0
D-045	29.1	0.2	0.2	**	0.2	0.1
D-046	78.5	0.1	0.3	0.1	0.1	0.0
D-048	7.5	0.1	0.1	0.1	0.0	0.1

* T cell lines were grown for a minimum of 3 weeks and the numbers indicate the % of CD8 T cells that produced IFNγ in response to the respective stimuli. ** not determined *** no cell line available Bold: % responding to an unrelated peptide is greater than % responding to negative control stimuli and the response of the negative control cell line

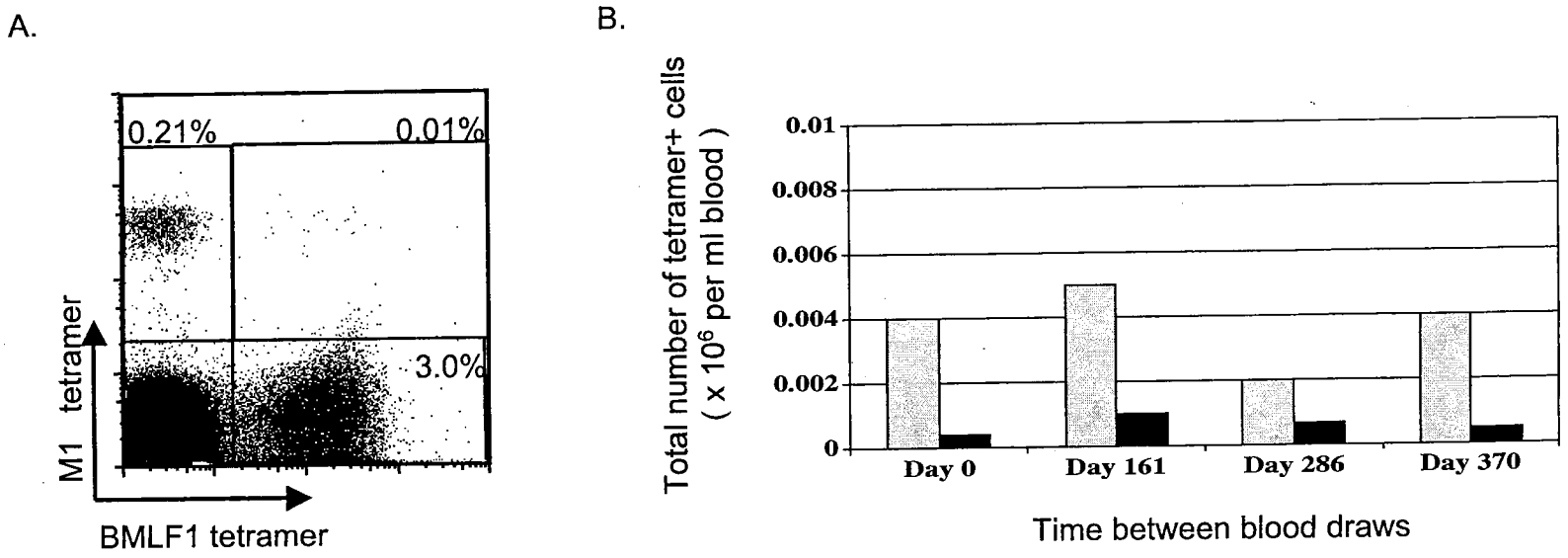


Figure 13. Stable, resting state, frequencies of antigen-specific T cells over time. CD8 T cells were isolated ex vivo from healthy donor D-002 and co-stained with M1- and BMLF1-loaded tetramers. (A) 10^6 events were collected to visualize double-tetramer staining cross-reactive cells. (B) The resting state frequency of tetramer positive T cells over time. (grey bars: BMLF1+, black bars: M1+)

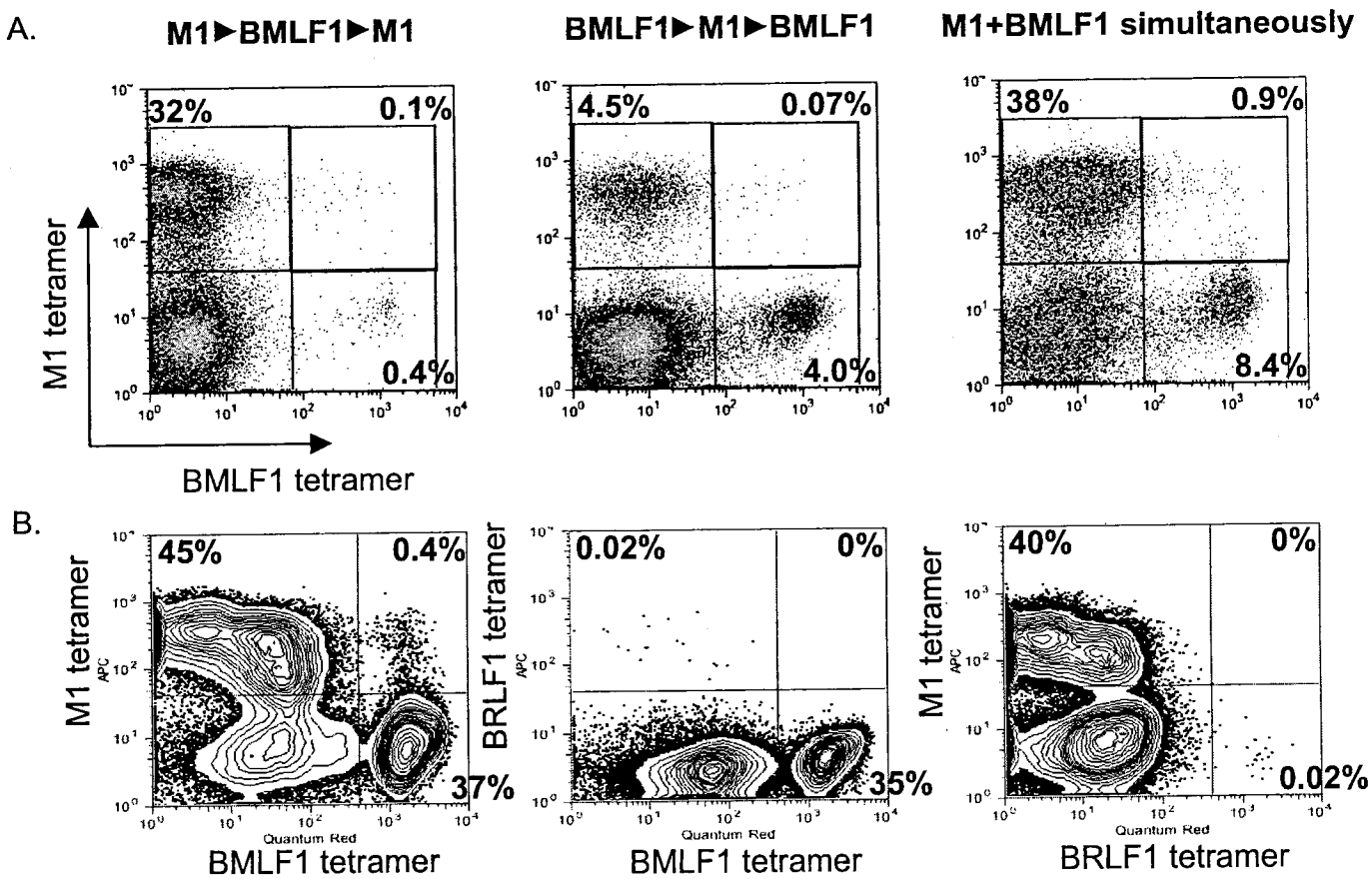


Figure 14. Culturing with M1 and BMLF1 peptides simultaneously promotes the growth of cross-reactive T cells. (A) CD8 T cell lines derived from healthy donor D-002 were cultured for 3 weeks, using the stimulating protocol shown above each plot (detailed in the text), and then were co-stained with M1- and BMLF1-loaded tetramers. (B) CD8 T cell line derived from the same donor was cultured for 4 weeks with a 1:1 ratio of M1 and BMLF1 peptide-pulsed T2 cells and then co-stained with tetramers. EBV-BRLF1-loaded tetramer served as a negative control stain.

and M1-loaded tetramers increased to a range of 0.3-1.1%, in the absence of any co-staining with a non-specific tetramer (Figure 14B and data not shown). The cross-reactive cells that bound both tetramers were also able to respond functionally to both epitopes. They produced MIP-1β, IFNγ and TNFα specifically following either M1 or BMLF1 stimulation, but BMLF1 stimulation appeared to result in a more robust production of all three cytokines (Figure 15A). However, a peptide titration assay revealed that these M1+ BMLF1+ cross-reactive T cells actually had a slightly higher avidity for the M1 epitope (Figure 16A). A concentration of 10^{-8} M M1 peptide, compared to a 10^{-7} M concentration of BMLF1 peptide, resulted in half of the maximum amount of IFNγ produced by this cross-reactive subset. The more robust functional response to BMLF1 initially observed using a 5 μM concentration of peptide appeared to be an effect of significant TCR downregulation, which decreased the sensitivity of M1 tetramer binding.

The tetramer-based frequency of cross-reactive cells within this T cell line was lower than the frequency based on function. The subset of cells only able to bind the M1-loaded tetramer produced MIP-1β and IFNγ, but very little TNFα, in response to BMLF1 stimulation (Figure 15B). The cells that bound only BMLF1-loaded tetramer also showed some degree of functional cross-reactivity. At least 35% of the BMLF1+ cells produced a low level of MIP-1β in response to M1 stimulation, although this cross-reactive stimulation was not as efficient at inducing IFNγ or TNFα production (Figure 15C). A separate peptide titration experiment on this single BMLF1 tetramer-positive subset revealed that the cross-reactive cells present in this sub-population had a similar

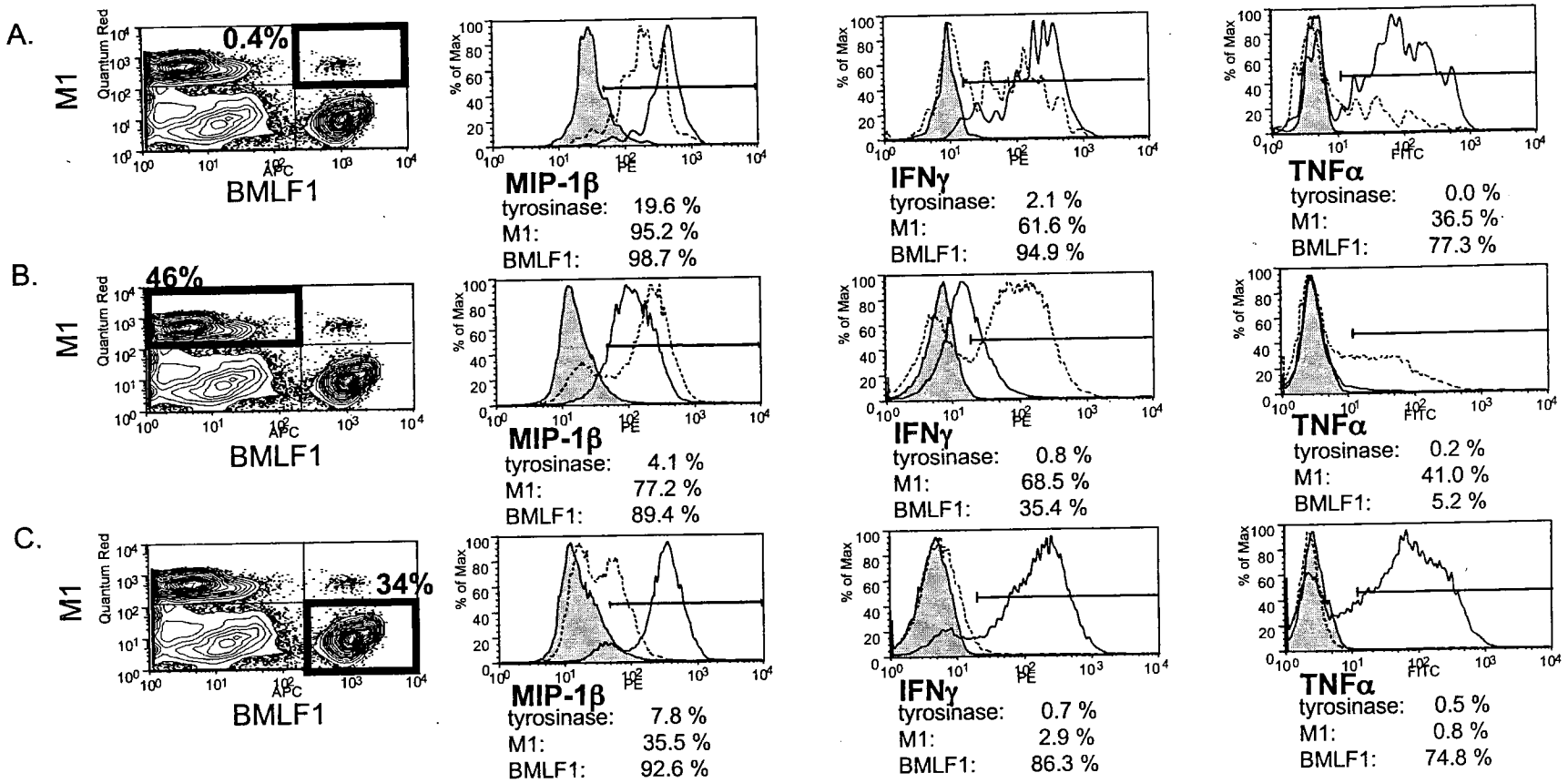
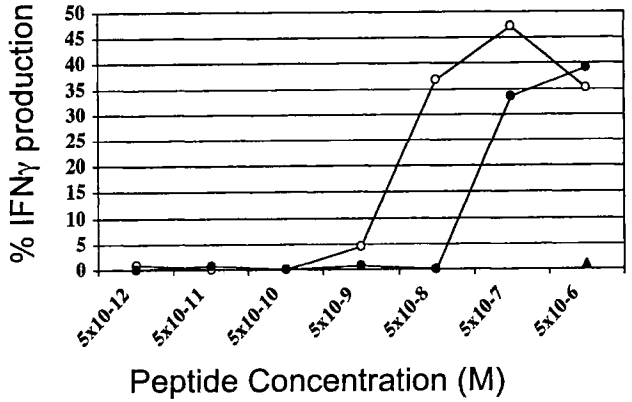


Figure 15. Three distinct cross-reactive populations within T cell lines cultured with M1 and BMLF1 simultaneously. A T cell line derived from healthy donor D-002 was grown for 4 weeks with a 1:1 ratio of M1 and BMLF1 peptide-pulsed T2 cells. We gated on (A) the % of cells staining with both M1 and BMLF1 tetramers, (B) the % of cells staining with only M1 tetramer, and (C) the % of cells staining with only BMLF1 tetramer before assessing cytokine production in response to the following peptide stimulations: tyrosinase (shaded), M1 (dotted line), BMLF1 (solid line). The % of the gated cells producing cytokine are shown below each histogram.

A. Double tetramer-positive subset



B. Single BMLF1 tetramer-positive subset

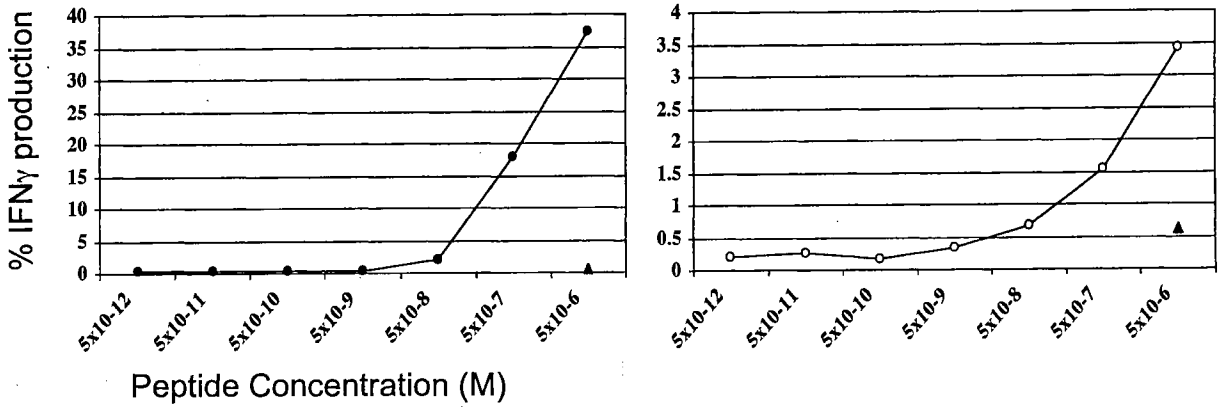


Figure 16. Tetramer-defined subsets of cross-reactive T cells differ in their avidity for the two peptides. Using a peptide titration, an intracellular IFNγ assay was performed on a T cell line derived from healthy donor D-002 that had been cultured with both M1 and BMLF1 peptides for 4 weeks. Analyses of the following tetramer-defined subsets of the T cell line are shown: (A) M1+ BMLF1+ and (B) M1- BMLF1+. (▲ tyrosinase, O M1, and ● BMLF1 stimulation)

avidity for the M1 and BMLF1 epitopes, where half the maximum amount of IFNγ was produced near a concentration of $5x10^{-7}$ M of either peptide (Figure 16B). The avidity remained high enough to stably bind the BMLF1 tetramer, but was insufficient for stable binding with the M1 tetramer. Overall, it would appear that the avidity of a cross-reactive T cell interaction with its alternative ligand is highly variable and that cross-reactive T cell populations are indeed heterogeneous. Hence, multiple techniques are required to detect T cell cross-reactivity, including tetramer staining and different functional assays. As shown here, TCR avidity is an important factor to consider when detecting cross-reactive T cell responses. An interaction between a cross-reactive T cell and its alternative ligand may be too weak to stably bind tetramer, but may still be sufficient to induce a distinct hierarchy of cytokine production. This is analogous to the observations that certain non-crossreactive influenza M1-specific clones were unable to bind M1-loaded tetramers but produced IFNγ following M1 peptide stimulation (Lawson et al., 2001).

We next sought to clone these cross-reactive cells from a polyclonal T cell line, using the experimental design outlined in Figure 17A. Briefly, we sorted a cell line with a relatively high population of cross-reactive cells and allocated single cells that co-stained with both BMLF1- and M1-loaded tetramers into micro-wells. The single cells were propagated for two weeks (referred to as clones from here on) and then assessed for functional specificity. As expected from the T cell line data, there was tremendous variability in the functional characteristics of each clone in response to either antigen. Of all the clones that grew, 8% produced IFNγ following stimulation with either M1 or

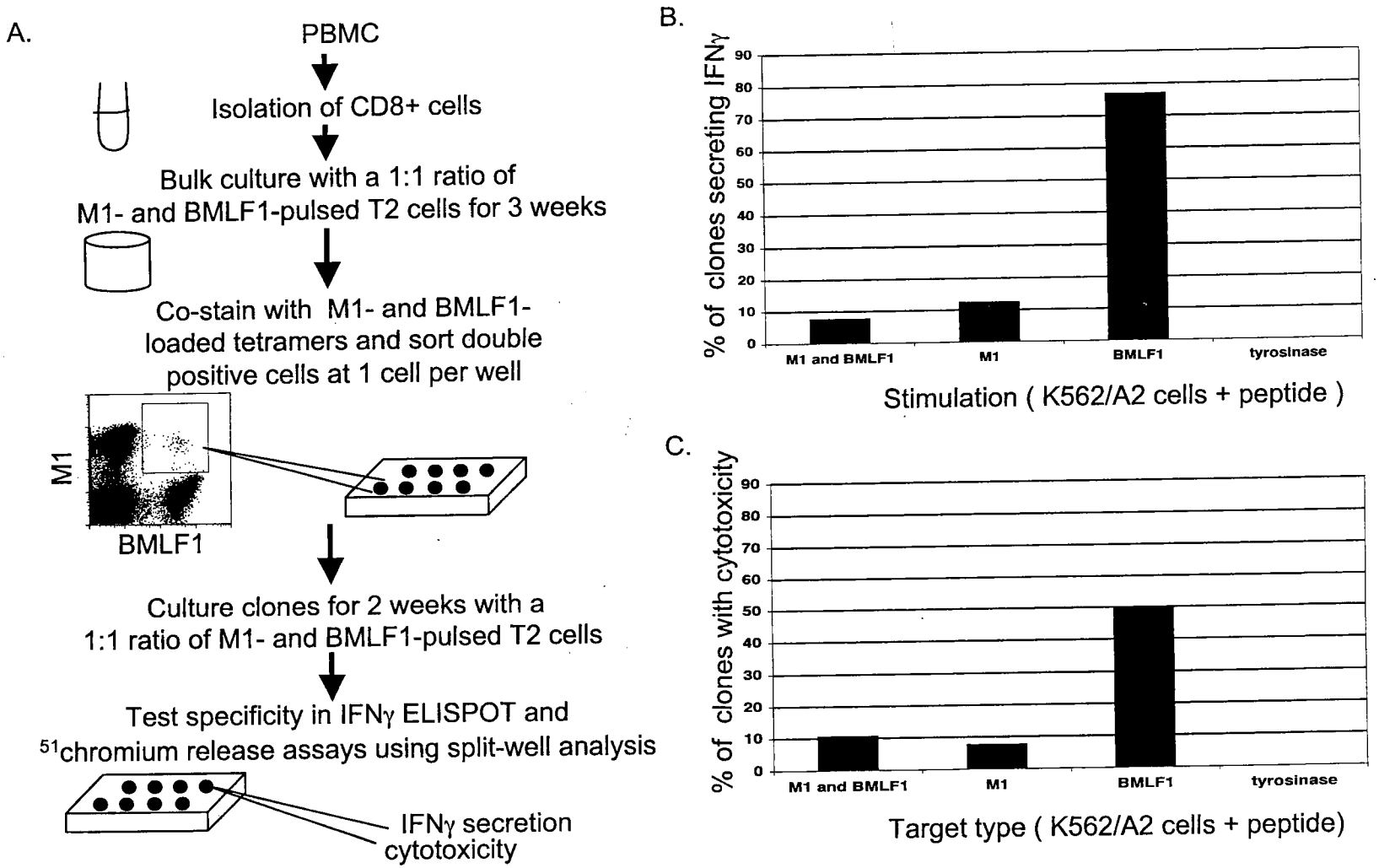


Figure 17. Cross-reactive clones are heterogeneous in their response to M1 versus BMLF1. (A) An outline of the experimental design used to clone cross-reactive T cells from healthy donor D-002. (B, C) The % shown on each bar graph represents the frequency of wells harboring T cells that (B) secreted IFNγ or (C) killed target cells following incubation with peptide-pulsed K562/HLA-A2 cells. Data presented here accurately represent the trends observed in three separate experiments.

BMLF1 (Figure 17B). Similarly, 11% of the different clones analyzed killed both M1- and BMLF1-pulsed target cells in a ^{51}chromium release cytotoxicity assay (Figure 17C). The number of functionally cross-reactive clones varied with the technique used for their detection and reflected a similar ratio of BMLF1-responders to M1-responders (4:1) as seen in the assessment of IFNγ production by the double-tetramer positive population within a polyclonal T cell line (1.5:1) (Figure 15A). These results definitively show that individual T cell clones can recognize and respond to both BMLF1 and M1, two epitopes which share little sequence similarity.

C. Cross-reactive T cells participate in the lymphoproliferation that defines the IM syndrome

Since we were able to detect cross-reactive cells that recognize M1 and BMLF1 in bulk culture and at a clonal level, we next sought to determine if these cross-reactive cells participate in the overzealous CD8 T cell response that defines EBV-asssociated IM. We noticed that, despite the large expansion of EBV-specific cells, the frequency (as percentage of CD8 T cells) of M1-specific cells in patients with IM (mean 0.20%, range: 0.02-0.49%) was similar to that of healthy influenza-immune donors (mean 0.26%, range: 0.09-0.79%). The maintenance of a resting-state frequency suggested that at least a subset of M1-specific cells was proliferating in response to infection, because non-crossreactive memory cells should be diluted out by the proliferation of virus-specific cells. In fact, the average number of M1-specific cells (per ml of blood) was a very significant 4-fold higher in patients with IM (0.004×10^6 per ml) compared to healthy donors (0.001×10^6 per ml) ($p= 0.02$) (Figure 18A). Five out of 8 patients with IM had a

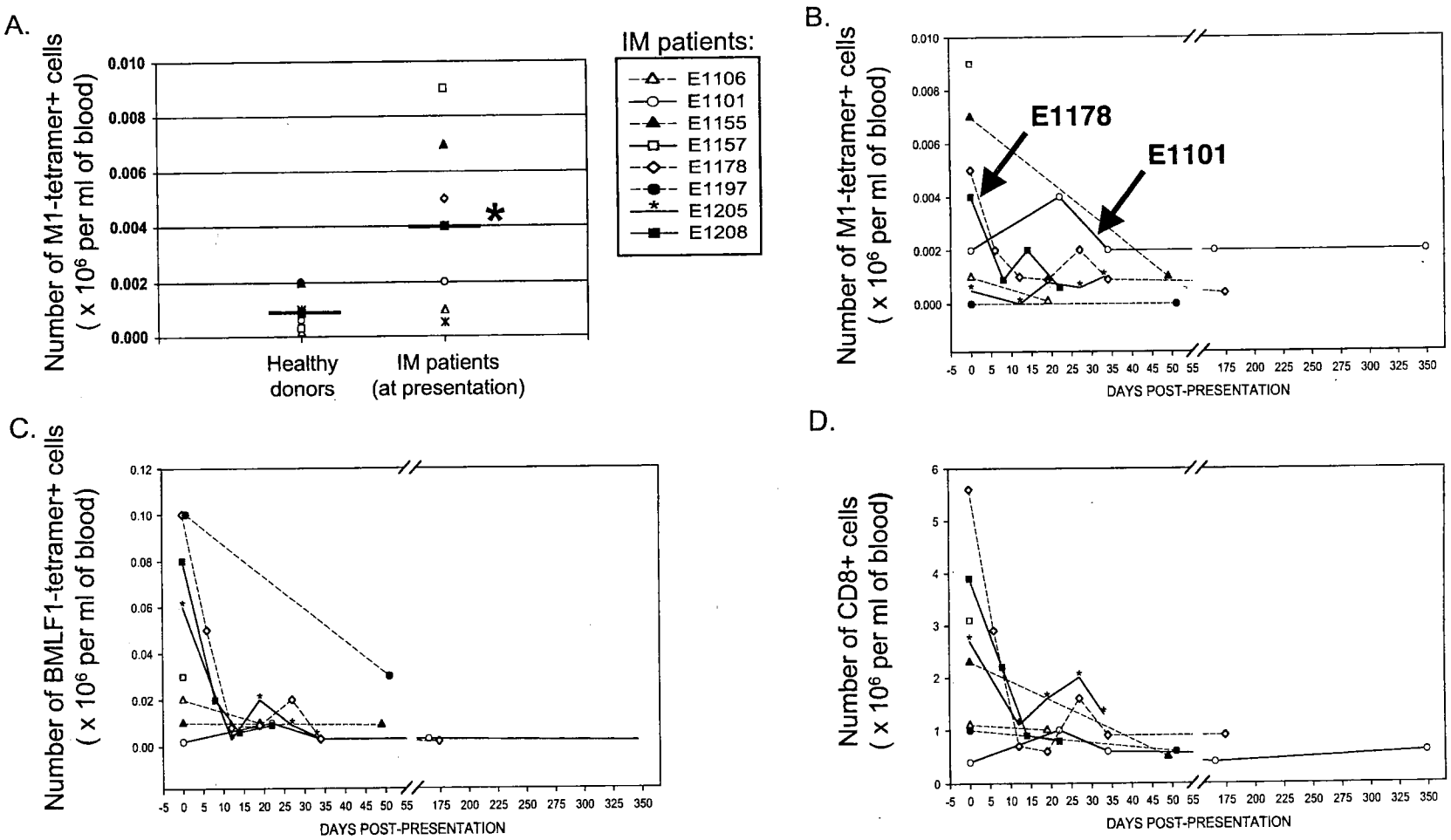


Figure 18. IM patients have an augmented number of M1-specific T cells in their bloodstream. PBMC were isolated from 8 healthy donors or from 8 IM patients. IM patient blood samples were collected at various points post-presentation with IM symptoms. Ex vivo tetramer stains on sorted CD8 T cells were used to determine the total number of (A, B) M1- or (C) BMLF1-specific cells per ml of blood. (A) The difference between the means was statistically significant using an unpaired, 2-tailed t-test (*p=0.02). Patient E1197 had an M1-specific memory population that grew out in culture, but was undetectable ex vivo; therefore, this patient was excluded from the calculation.

higher than average number of M1-specific cells at presentation, and this number decreased over the course of the infection with contraction kinetics similar to the BMLF1 response and the overall lymphoproliferation (Figure 18B-D).

These observations suggested that acute EBV infection can activate influenza-specific memory cells through a TCR-dependent mechanism. The expansion of M1-specific memory cells was evident in only 5 of the 8 IM patients examined despite the fact that all had memory to M1, and all would have been influenced by any cytokine-mediated, or bystander, activation. When possible, we also looked for the expansion of a second memory T cell population, specific for CMV-pp65. Only two IM patients proved to be CMV seropositive: E1155 and a recent enrollee, E1238. The frequency of pp65-specific T cells in patients E1155 and E1238 dropped during the massive, EBV-induced, lymphoproliferation. At day 0, E1155 and E1238 had a pp65-specific T cell frequency of 0.2% and 0.7% respectively while, by 41-50 days post-presentation, those frequencies climbed to 0.6% and 1.1% respectively. These data would suggest that the pp65-specific memory populations of these two patients did not contain T cell clones cross-reactive with EBV and were, therefore, initially diluted out by the extensive proliferation of EBV-specific T cells.

In an attempt to determine the cross-reactive specificity of the augmented M1-specific T cell populations, we co-stained freshly isolated CD8 T cells from IM patients with M1- and BMLF1-loaded tetramers. Double-positive cross-reactive cells were prominent in two patients, E1101 and E1178 (Figure 19). The percentage, as well as the total number, of cross-reactive cells shifted with this active infection, including a

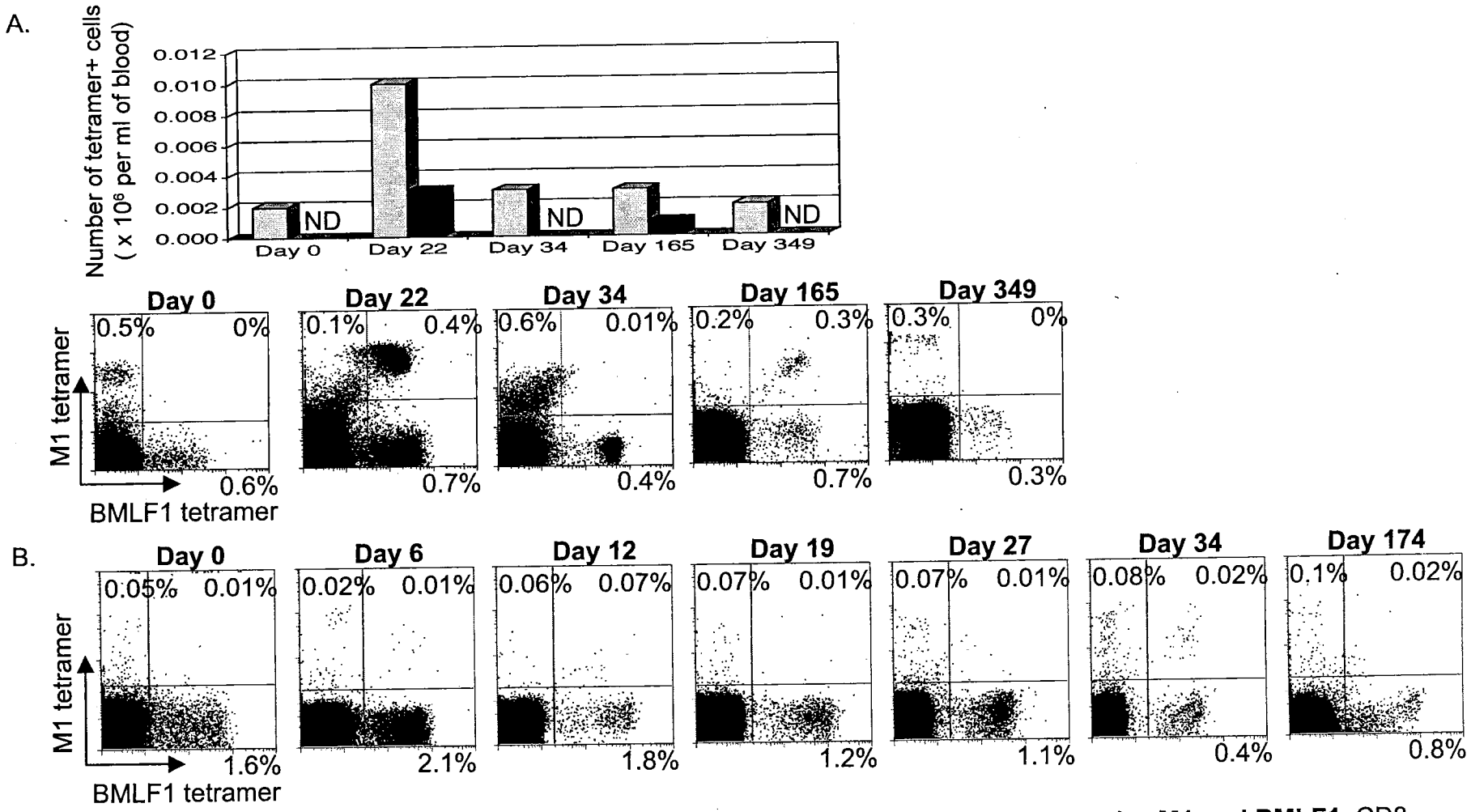


Figure 19. Acute EBV infection augments the number of cross-reactive cells that recognize M1 and BMLF1. CD8 T cells were isolated ex vivo from patient (A) E1101 or (B) E1178 at various time points post-presentation with IM symptoms. The percentages of CD8 T cells staining positive when co-stained with M1- and BMLF1-loaded tetramers are shown. The number of events shown is variable because the maximum number possible was collected for each sample. (A) The total number of BMLF1-specific and cross-reactive T cells per ml of blood was calculated using tetramer frequencies (BMLF1+: grey bars, BMLF1+ M1+: black bars, ND: not detectable).

considerable increase at day 22 (0.003 x 10^6 M1+ BMLF1+ cells per ml of blood) post-presentation of patient E1101's clinical symptoms (Figure 19A). This translated to as many as 1/3 of the T cells specific to the immunodominant BMLF1 epitope being cross-reactive with M1. Fewer tetramer-defined cross-reactive T cells were detected during patient E1178's infection, but there was a discernable increase in frequency at days 12 and 34 (0.0002 x 10^6 M1+ BMLF1+ cells per ml of blood) post-presentation with IM (Figure 19B). Both of these patients presented with symptoms of IM but with differences in regards to the severity. Patient E1101, who had the higher frequency of M1+ BMLF1+ cross-reactive cells, presented with severe (grade 5 on a scale of 1-5) symptoms and clinical signs of IM, including fatigue, sore throat, nausea, myalgia, lymphadenopathy, pharyngitis, and stomatitis (Table 5A). Notably, reversion of the CD4:CD8 T cell ratio was not evident until day 34 post-presentation (Table 5B). In contrast, patient E1178, who had a lower frequency of cross-reactive cells, presented with only moderate (grade 2-3) symptoms and clinical signs of IM, including fatigue, loss of appetite, and only mild hepato-splenomegaly (Table 5A). For this patient, CD4:CD8 reversion was observed on days 0 and 6, with a noticeable decrease in ratio again at day 27 post-presentation (Table 5B). The slight increase in CD8 T cell frequency at day 27 was synonymous with a slight increase in viral load, which may have been enough to alter the hierarchy of responding T cell clones and result in the proliferation of cross-reactive T cells detected later during the immune response (Figure 20). More patients will need to be studied to determine whether the correlation between cross-reactivity with M1 and disease severity will hold, but here we document high levels of cross-reactive T

Table 5. Severity of IM in patients having a cross-reactive T cell response specific for M1 and BMLF1.

A.

	Patient E1101:			Patient E1178:		
Symptoms	**Present**	**Duration***	**Severity****	**Present**	**Duration***	**Severity****
Malaise	Y	7	5	Y	14	2-3
Sweats	Y	7	5	N		
Sore throat	Y	7	5	N		
Loss of appetite	Y	7	5	Y	14	2-3
Nausea	Y	7	5	N		
Headache	N			N		
Chills	Y	7	5	Y	occas.	
Myalgia	Y	7	5	N		
Signs	**Present**	**Duration***	**Severity****	**Present**	**Duration***	**Severity****
Adenopathy	Y	7	5	N		
Fever	N			N		
Pharyngitis	Y	7	5	N		
Periorbital edema	N			N		
Hepatomegaly	N			Y	14	1-2
Splenomegaly	N			Y	14	1-2
Stomatitis	Y	4	5	N		

* Duration in days, ** Grade of severity from 1 (mild) to 5 (severe)

B.

Patient E1101:			Patient E1178:	
Day***	**CD4:CD8**		**Day*****	**CD4:CD8**
0	3.6		0	0.3
22	1.7		6	0.5
34	0.9		12	1.1
165	1.9		19	1.2
349	3.2		27	1.0
			34	1.4
			174	1.7

*** Days post-presentation with IM symptoms

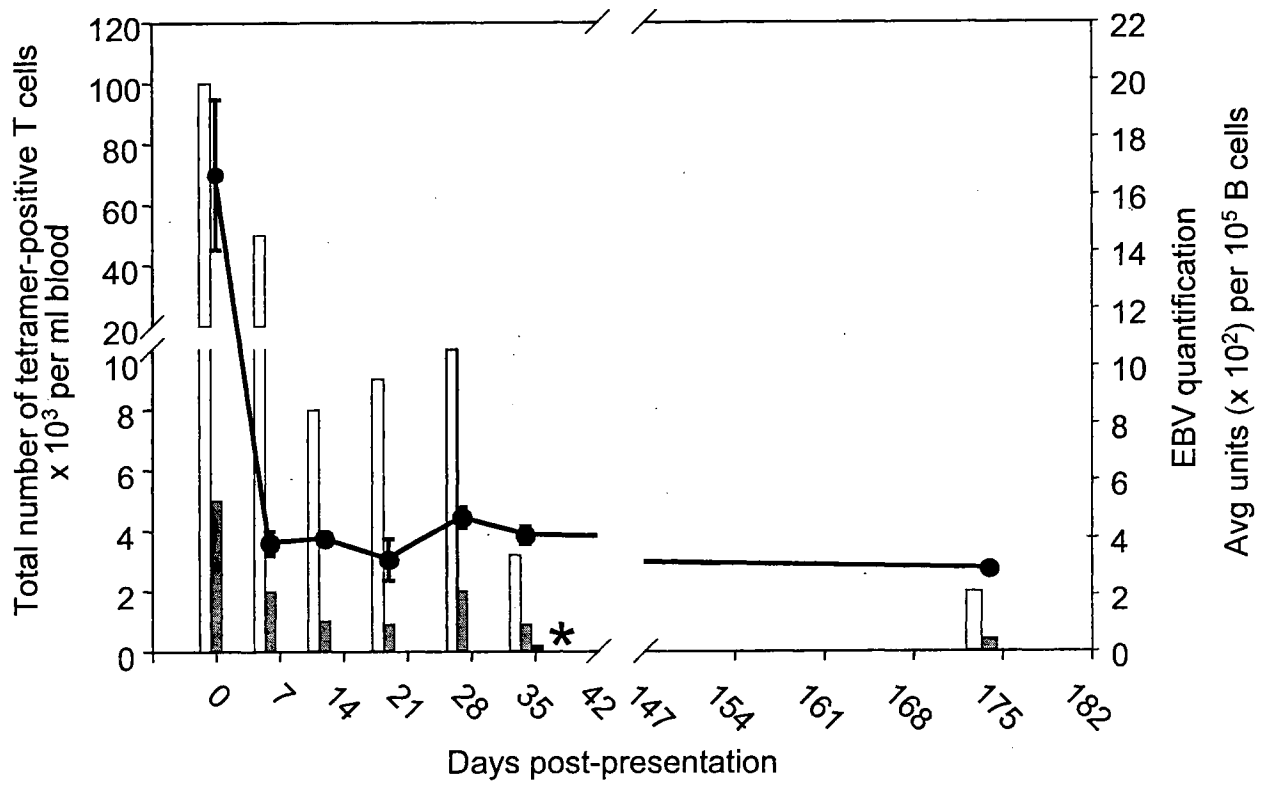


Figure 20. Frequency of antigen-specific T cells in the context of viral load. PBMC were isolated from patient E1178 at various time points post-presentation with IM symptoms and were separated into CD8 positive and negative fractions. CD8+ cells were co-stained with tetramers and the frequencies used to calculated the total number of BMLF1+ (white bars), M1+ (grey bars), and BMLF1+ M1+ (*black bars) cells per ml of blood (left axis). CD8- cells were stained for CD19+ B cells, and the DNA of 10^5 B cells was used to quantify the level of EBV (line graph, right axis).

cells associated with severe IM pathology.

D. Skewing of the M1-specific Vβ17+ TCR repertoire during IM

The M1-specific memory TCR repertoire is organized in a conserved pattern, as unique TCR clonotypes using the Vβ17 gene family can be ordered in a distinct hierarchy based on their Jβ usage, where Jβ2.7 dominates (used by 55-62% of clonotypes), followed by Jβ2.3 (used by 10-20% of clonotypes) and then often Jβ2.1, Jβ2.5, Jβ1.1, or Jβ1.2 at lower, more variable, frequencies (Figure 21A) (Naumov et al., 2005). We investigated whether the M1-specific repertoires of E1101 and E1178 were skewed from this pattern and thereby reflective of cross-reactive TCR-mediated expansions. Due to limited blood samples, we were unable to sort and sequence the TCRs of the M1-specific cells directly ex vivo but, instead, generated M1-specific T cell lines from these IM patients. Vβ analyses indicated that the M1-specific repertoires of patients E1101 and E1178 were focused on the Vβ17 family, similar to that previously described for healthy individuals (data not shown) (Lawson et al., 2001; Lehner et al., 1995; Moss et al., 1991; Naumov et al., 2003). However, when we sequenced the Vβ17+ clonotypes within the cell lines derived from both patients, they did not follow the highly conserved organizational pattern observed in healthy IV-immune donors. At day 22 post-presentation, the time point when the number of cross-reactive T cells was highest ex vivo, the Jβ2.3 family was over-represented (30%) while the normally dominant Jβ2.7 family was vastly under-represented (10%) within the M1+ Vβ17+ repertoire of patient E1101 (Figure 21B). The skewing of the repertoire was even more pronounced at day

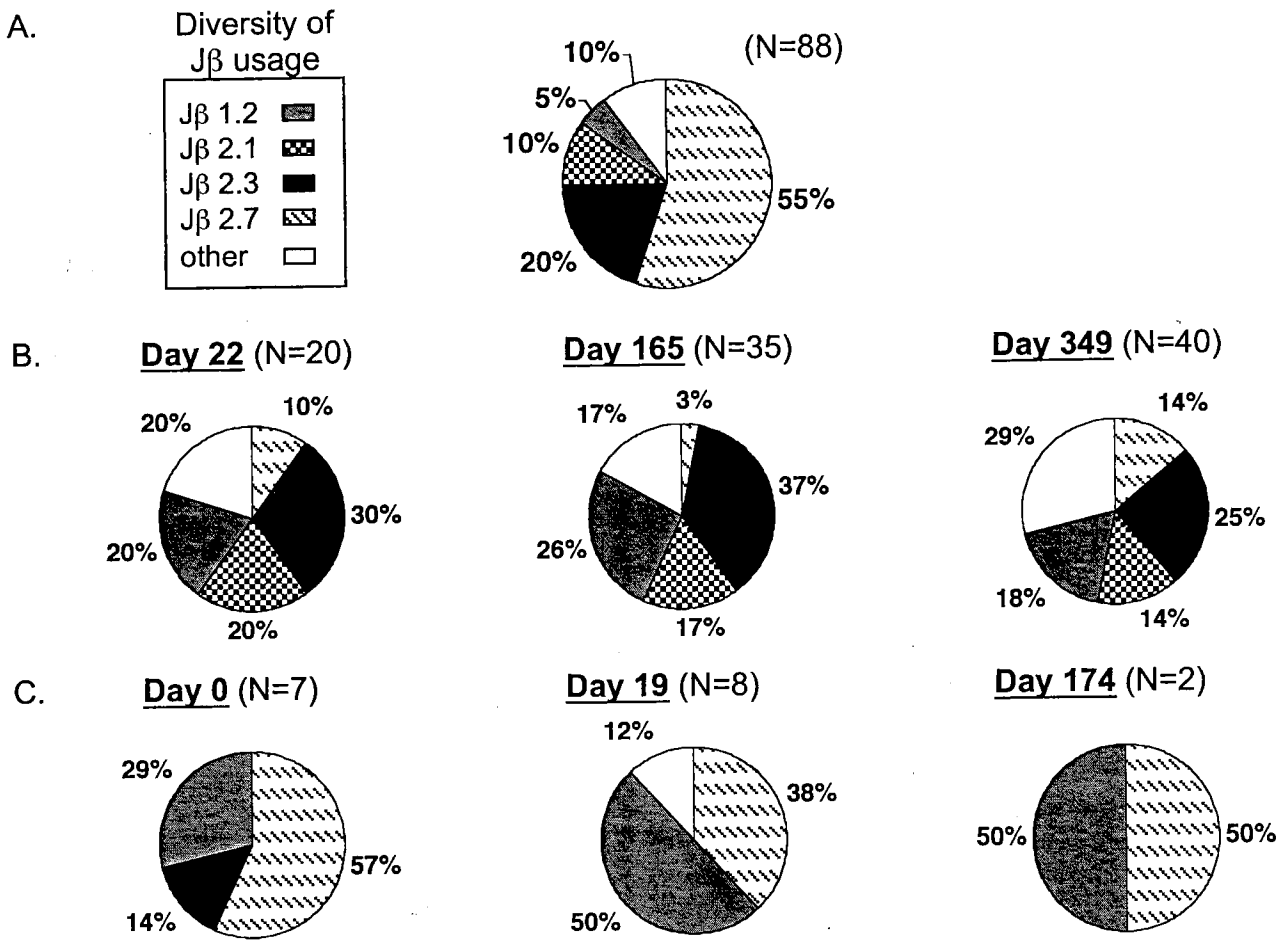


Figure 21. Acute EBV infection skews the M1-specific TCR repertoire. M1-specific T cell lines, derived from (A) healthy donor D-002 or IM patients (B) E1101 and (C) E1178 at various time points post-presentation with IM symptoms, were cultured for 3 weeks. Total RNA was isolated from these T cell lines, cDNA was synthesized, and the CDR3β regions of Vβ17+ T cells were sequenced. The pie charts illustrate the percentage of unique Vβ17+ clonotypes using each Jβ family, where N = the total number of unique clonotypes. The complete CDR3 sequence of all the clonotypes analyzed are displayed in Table 6.

Table 6. Vβ17+ TCR repertoire of the M1-specific T cell lines derived from IM patients

A. IM patient: E1101

M1 line	[1]Length:	[2]ID:	Freq:	JB:	[3]AA seq of CDR3β loop:	Nucleotide sequence of the V/N/D/N/J junction:
Dav 22	7 AA	H2.1	1	1.2	AS-SIGLYGY-TF	gccagtagtattggtctctatggctacacctc
		H2.2	1	1.2	AS-SIGYYGY-TF	gccagtagtatcgggtactatgggtacacctc
		H2.3	1	1.2	AS-STGWYGY-TF	gccagtagtgccgggtgtctatgggtacacctc
		H4.3	1	1.2	AS-SMGSYGY-TF	gccagtagtatgggttcctatgggtacacctc
	8 AA	H4.10	7	1.3	AS-SIRSGNTI-YF	gccagtagtattcgttcgggaaacaccatatatttt
		H2.6	4	2.1	AS-STRAGWEQ-FF	gccagtagtacacgggcgggggtgagcagttcttc
		H4.24	2	2.3	AS-SGRSADTQ-YF	gccagtagcgggacggcgcgcagatacgcagttttt
		H2.10	2	2.3	AS-SRRSTDTQ-YF	gccagtagtcgacggagcacagatacgcagttttt
		H2.4	1	1.6	AS-SIGYNSPL-HF	gccagtagtatcgggctaatttcacccctccacttt
		H2.5	1	2.1	AS-SIRSGYEQ-FF	gccagtagtatacgatcgggtatgtagcagttctc
		H2.7	1	2.1	AS-SAGLASEQ-FF	gccagtagtgcgcggactttgctagtgagcagttctc
		H2.8	1	2.3	AS-SIRSTDTQ-YF	gccagtagtatacggagcacagatacgcagttttt
		H5.19	1	2.3	AS-SIRSTDTQ-YF	gccagtagtatacaggagcacagatacgcagttttt
		H2.9	1	2.3	AS-SGRSTDTQ-YF	gccagtagcgggacggagcacagatacgcagttttt
		H2.11	1	2.5	AS-SNRAGETQ-YF	gccagtagtaacaggcagggcagaggagacccagtactc
		H2.12	1	2.7	AS-SIRSSTEQ-YV	gccagtagtattaggctatagcacggagcagtacgtc
		H2.13	1	2.7	AS-SVRSAYTQ-YF	gccagtagtgtccggagtgcttatacgcagttttt
	9 AA	H2.14	1	2.6	AS-SPRSGANVL-TF	gccagtagtcccccttctcgggcccaacgtcctgacttttc
		H2.15	1	2.3	SS-SSRSGEDTQ-YF	tccagtagtagcccgatcgggcagggatacgcagttttt
	11 AA	H2.16	1	2.1	AS-SIVMGSSYNEQ-FF	gccagtagtatagtaatgggagcgctcctacaatgagcagttctc
Dav 165	7 AA	H4.1	6	1.2	AS-SIGSYGY-TF	gccagtagtatcggatcgtacgggctacacctc
		H4.3	5	1.2	AS-SMGSYGY-TF	gccagtagtatgggttcctatgggtacacctc
		H4.2	1	1.2	AS-SIGSNGY-TF	gccagtagtatcggatcgaacgggtacacctc
		H4.4	1	1.2	AS-SMGSYGY-TF	gccagtagtatgggttcctatggttacacctc
		H4.5	1	1.2	AS-SIGILHGY-TF	gccagtagtatcaggcctcatgggtacacctc
		H4.6	1	1.2	AS-SIGIHGY-TF	gccagtagtatcaggatccacgggtacacctc
		H4.7	1	1.2	AS-SIGVHGY-TF	gccagtagtatcgtgggtccatgggtacacctc
		H4.8	1	1.2	AS-STGLYGY-TF	gccagtagtaccgggttatatgggtacacctc
		H4.9	1	1.2	AS-STGWYGY-TF	gccagtagccacagggattttatgggtacacctc
	8 AA	H4.13	9	2.3	AS-SIRSGDTQ-YF	gccagtagtatacggagtggagatacgcagttttt
		H4.10	7	1.3	AS-SIRSGNTI-YF	gccagtagtattcgttcgggaaacaccatatatttt
		H4.19	4	2.3	AS-SPRSTDTQ-YF	gccagtagtccacggagccacgatacgcagttttt
		H4.11	2	2.1	AS-STRHSNEQ-FF	gccagtagtacctttccacgccaatgagcagttctc
		H4.21	2	2.3	AS-SGRSTDTQ-YF	gccagtagccaaggatcccacgatacgcagttttt
		H4.12	1	2.1	AS-SLVSHNEQ-FF	gccagtagtctagtatcccacaatgagcagttctc
		H4.20	1	2.3	AS-STRSTDTQ-YI	gccagtagtactaggagccacgatacgccgtgtatmh
		H4.17	1	2.3	AS-SMRSTDTQ-YF	gccagtagtatcgcggagccacgatacgcagttttt
		H4.18	1	2.3	AS-SMRSTDTQ-YF	gccagtagtatcgcggagccacgatacgcagttttt
		H4.14	1	2.3	AS-SIRSTDTQ-YF	gccagtagtatcacggagccacgatacgcagttttt
		H4.16	1	2.3	AS-SGRSTDTQ-YF	gccagtagccgggcgtagccacgatacgcagttttt
		H4.15	1	2.3	AS-SGRSTDTQ-YF	gccagtagtcgccggagccacgatacgcagttttt
		H4.22	1	2.3	AS-SLRSTDTQ-YF	gccagtagcctaagatccacgatacgcagttttt
		H4.23	1	2.3	AS-SIVSTDTQ-YF	gccagtagtatagtcagcacgatacgcagttttt
		H4.24	1	2.3	AS-SGRSADTQ-YF	gccagtagcggacggagcgccgatacgcagttttt
		H4.25	1	2.3	AS-SVRSAYTQ-YF	gccagtagtgtccggagtgcttatacgcagttttt
		H4.26	1	2.5	AS-SGRASETQ-YF	gccagtagccggagcgccctcagagacccagtactc
		H4.27	1	2.5	AS-SMRSGETQ-YF	gccagtagtatcgcgatcgcgggcagcccagtactc
		H4.28	1	2.7	AS-SIRSSYEQ-YF	gccagtagtattaggagctcctacgacgtactc
	9 AA	H4.29	2	1.5	AS-SIFSGDQPQ-HF	gccagtagtatttctacgggacccagcccacgcatttt
		H4.30	1	2.1	AS-STVGGINEQ-FF	gccagtagtacgggtgggtggaatcaatgagcagttctc
		H4.31	1	2.4	AI-LRGSGVHIQ-YF	gccatcctacgggtgagcggagtacacattcagtactc
	10 AA	H4.32	1	2.1	AS-SIGASDYNEQ-FF	gccagtagtatcagggctagcgactacaatgagcagttctc
		H4.33	1	2.1	AS-SQGGINFYNEQ-FF	gccagtagccagggggtgaatttttacaatgagcagttctc
	11 AA	H4.34	1	2.1	AS-SIVGGRWGNEQ-FF	gccagtagtatagtgggcagggtgggggaatgagcagttctc
		H4.35	1	2.2	AS-SIEPSRNTGEL-FF	gccagtagtatgagcgcctagtcgaaacaccgggcagctgttttt
Dav 349	6 AA	5.1	1	2.1	AS-SIWDEQ-FF	gccagtagtatatgggatgaccagttctc
	7 AA	4.6	2	1.2	AS-SIGIHGY-TF	gccagtagtatcagggatccacggctacacctc
		4.1	2	1.2	AS-SIGSYGY-TF	gccagtagtatcggatcgtacgggctacacctc
		4.3	1	1.2	AS-SMGSYGY-TF	gccagtagtatgggttcctatgggtacacctc
		5.30	1	1.2	AS-GMGSYGY-TF	gccagtggtatgggatcctatgggtacacctc
		5.2	1	1.2	AS-SIGLYGY-TF	gccagtagtatcggcctctatgggtacacctc
		5.3	1	1.2	AS-SIGLYGY-TF	gccagtagtatcgggctctatgggtacacctc
		4.8	1	1.2	AS-STGLYGY-TF	gccagtagtacgggcttatatgggtacacctc
		5.4	1	1.2	AS-SIGSYGY-TF	gccagtagtatcaggctctatgggtacacctc
	8 AA	4.10	9	1.3	AS-SIRSGNTI-YF	gccagtagtattcgttcgggaaacaccatatatttt
		5.9	6	2.7	AS-SVRSSYEQ-YF	gccagtagtgtccgggagctcctacgacgtactc
		5.19	5	2.3	AS-SIRSTDTQ-YF	gccagtagtatcaggagccacgatacgcagttttt
		4.28	4	2.7	AS-SIRSSYEQ-YF	gccagtagtattaggagctcctacgacgtactc
		4.24	3	2.3	AS-SGRSADTQ-YF	gccagtagccgacggagcgcagatacgcagttttt
		5.11	2	1.5	AS-SIRSNQPQ-HF	gccagtagtattcggtcaatcagcccacgcatttt
		4.13	2	2.3	AS-SIRSGDTQ-YF	gccagtagtatacggagtggagatacgcagttttt
		5.9	2	2.7	AS-SIRSSYEQ-YF	gccagtagtattaggagctcctacgacgtactc
		5.5	1	1.3	AS-SIRSGNTI-YF	gccagtagtattcgttcgggaaacaccatatatttt
		5.6	1	1.6	AS-SIRSSNPL-HF	gccagtagtattaggtcgtcgaatcccctccacttt
		5.20	1	2.1	AS-SIMSLDEQ-FF	gccagtagtatcaatgagcttggatgagcagttctc
		5.17	1	2.2	AS-SIRSTGEL-FF	gccagtagtatacgggagccacccgggagctgttttt
		4.25	1	2.3	AS-SVRSAYTQ-YF	gccagtagtgtccggagtgcttatacgcagttttt
		5.14	1	2.3	AS-SGRSTDTQ-YF	gccagtagtcggtagtagcgacacgatacgcagttttt
		5.15	1	2.3	AS-STRSTDTQ-YF	gccagtagtacccgtagcacacgatacgcagttttt
		5.16	1	2.3	AS-STRSTDTQ-YF	gccagtagtcctagtgtcacacgatacgcagttttt
		4.19	1	2.3	AS-SPRSTDTQ-YF	gccagtagtccacggagcacgatacgcagttttt
		5.18	1	2.3	AS-SIRSTDTQ-YF	gccagtagtatcgggagcacgatacgcagttttt
		4.21	1	2.3	AS-SQRSTDTQ-YF	gccagtagccaacgatccacgatacgcagttttt
		4.27	1	2.5	AS-SMRSGETQ-YF	gccagtagtatcgcgatcgcgggcagacccagtactc
		4.26	1	2.5	AS-SGRASETQ-YF	gccagtagccggagcgccctcagagacccagtactc
		5.7	1	2.7	AS-SIRSSYEQ-YF	gccagtagtattaggagctcctacgacgtactc
		5.8	1	2.7	AS-SIRSSYEQ-YF	gccagtagtatccgaagctcctacgggcagtactc
		5.12	1	2.7	AS-SIRASYEQ-YV	gccagtagtatcccgggcttcatcacgacgtacgtc
	9 AA	5.21	2	1.1	AS-SATIGNTEA-FF	gccagtagtgcggacgattgggaacactgaagcgttcttt
		4.30	2	2.1	AS-STVGGINEQ-FF	gccagtagtacggtgggtggaatcaatgagcagttctc
		5.24	1	1.3	AS-SIRSGNTK-YL	gccagtagtattcgttcgggaaacaccaacaatattnggga
		5.22	1	2.1	AS-SSSTSNEQ-FF	gccagtagtacgacgtaccctacaatgagcagttctc
		5.23	1	2.2	AS-SARASLGEL-FF	gccagtagtgccagggcgagcctcgggagcgttttttt
	10 AA	5.27	1	1.1	AS-SIHGGGNTEA-FF	gccagtagtatccacggtggtgcgaaacactgaagcttctctt
		4.33	1	2.1	AS-SQGGINFYNEQ-FF	gccagtagccagggggtgaatttttacaatgagcagttctc
		5.25	1	2.2	AS-RVLAGPAGEL-FF	gccagtagggttctacgcgggcccgccgcgggagcgttttt
		5.26	1	2.3	AS-SITRLSTDTQ-YF	gccagtagtatcaccggattaagcacagatacgcagttttt
	12 AA	5.28	3	2.1	AS-SRLAGDAWYNEQ-FF	gccagtagtagactagcgggagatgcctgggtacaatgagcagttctc
	13 AA	5.29	2	2.5	AS-SISPSVFREETQ-YF	gccagtagtattagccatcggtctttcgggaggagagaccgagtactc

B. IM patient: E1178

M1 line	[1]Length:	[2]ID:	Freq:	JB:	[3]AA seq of CDR3β loop:	Nucleotide sequence of the V/N/D/N/J junction:
Dav 0	7 AA	v1m8	4	1.2	AS-SIGSYGY-TF	gccagtagtatcaggcagctatggctacacctc
		v1m9	1	1.2	AS-SIGVYGY-TF	gccagtagtatcagggtgtctatggctacacctc
	8 AA	v1m2	5	2.7	AS-SIRSSYEQ-YF	gccagtagtatacaggagctcctacgacgtactc
		v1m5	2	2.7	AS-STRSSYEQ-YF	gccagtagtaccaggagctcctacgagcagtactc
		v1m4	1	2.3	AS-SIRSTDTQ-YF	gccagtagtatccgggagcacagatacgcagttttt
		v1m11	1	2.7	AS-SIRSSYEQ-YF	gccagtagtatacaggagctcctacgagcagtactc
		v1m15	1	2.7	AS-SIRSSYEQ-YF	gccagtagtatccgcagttcctacgagcagtactc
Dav 19	7 AA	v4m7	2	1.2	AS-SIGVYGY-TF	gccagtagtatcagggtgtctatggctacacctc
		v4m1	1	1.2	AS-SIGSYGY-TF	gccagtagtatcggaagttatggctacacctc
		v4m2	1	1.2	AS-SIGSYGY-TF	gccagtagtattgggagctatggctacacctc
		v4m20	1	1.2	AS-SIGCYGY-TF	gccagtagtwttggtgttatggctacacctc
	8 AA	v4m3	8	2.7	AS-SIRSAYEQ-YF	gccagtagtatccgttcggccctacgagcagtactc
		v4m4	2	2.7	AS-SIRSSYEQ-YF	gccagtagtatacaggagctcctacgagcagtactc
	9 AA	v4m5	1	2.7	AS-SITSGGINEQ-YF	gccagtagtatacactagcgggggcaacgagcagtactc
		v4m6	1	1.1	AS-SLGRVHTEA-FF	gccagtagtcgcggcagggtgcacactgaagcttctctt
Dav 174	7 AA	v7m2	10	1.2	AS-SIGIYGY-TF	gccagtagtatcgggatctatggctacacctc
	8 AA	v7m1	9	2.7	AS-STRSSYEQ-YF	gccagtagtacgaggagctcctacgagcagtactc

[1] Length of CDR3β loop according to Chothia et al., shown supported by 2 flanking framework regions [2] Each clonotype is distinguished by a unique nucleotide sequence at the V/N/D/N/J junction [3] Bolded residues correspond to the N/D/N region of the CDR3β loop.

165 (Jβ2.3=37%, Jβ2.7=3%), again a time when cross-reactive cells could easily be detected ex vivo (Figure 21B). When the M1-specific population was presumably more stable at 1 year post-presentation, its repertoire was still skewed from that typical of a resting memory state but appeared to be slowly reverting, as the number of clonotypes using Jβ2.3 (25%) declined and those using Jβ2.7 (14%) were better represented (Figure 21B). Similarly, the M1-specific repertoire of patient E1178 was skewed during the acute phase of the immune response to EBV, but in a way that was unique from patient E1101. In the case of patient E1178, we found that the Jβ1.2 family was over-represented within the M1+ Vβ17+ repertoire (50% at days 19 and 174), illustrating the point that a cross-reactive TCR repertoire may be unique to each individual and therefore not easily predicted (Figure 21C). This perturbation in the (IV)M1-specific TCR repertoire of both patients with infectious mononucleosis is highly consistent with the concept that the cross-reactive M1-specific T cells are expanded in the host in an antigen-driven manner by EBV-derived epitopes, such as BMLF1. While we did not have access to their blood prior to EBV infection, the M1+ Vβ17+ repertoires of both IM patients were markedly skewed from the conserved organizational pattern of M1+ Vβ17+ repertoires of healthy individuals with long-term immunity to both EBV and IV.

E. Chapter IV summary

We show here that cross-reactive T cells specific to a previously encountered virus, such as IV, could be major contributors to the overzealous CD8 T cell response that defines infectious mononucleosis. In 5 out of 8 patients, (IV)M1-specific CD8 T cells participated in the EBV-induced lymphoproliferation. Two of these 5 patients had

dramatically skewed M1-specific TCR repertoires and increased levels of clearly identifiable, tetramer-defined, cross-reactive CD8 T cells capable of recognizing the two dissimilar epitopes (IV)M1 and (EBV)BMLF1. Based on our ability to culture these cross-reactive cells from 3 out of 8 healthy donors with previous exposure to both viruses, these cross-reactive cells are maintained in memory and their functional responses to either antigen can include cytotoxicity and the production of MIP-1β, IFNγ and TNFα. Cross-reactive T cells may play a major role in the development of IM, and the diversity of their functions may contribute to the severity of the syndrome. These studies examined only one cross-reactive population, while it is likely that infection with EBV, a virus with the potential to encode hundreds of epitopes, could reactivate many memory T cell populations yet undefined. As demonstrated here, the identification of cross-reactive T cells can be complicated by their ability to recognize alternative peptides having little sequence similarity to the native peptide, their strict growth requirements in vitro, and the sensitivity of the different techniques used to detect them. These are all challenges for future elucidations of individual cross-reactive T cell responses and their potential impact on the outcome of infectious mononucleosis.

CHAPTER V:

THE CLONAL COMPOSITION OF THE CROSS-REACTIVE CD8 T CELL RESPONSE WITH SPECIFICITY FOR EBV-BMLF1$_{280-288}$ AND IV-M1$_{58-66}$: FEATURES OF A CROSS-REACTIVE TCR REPERTOIRE

Our data showed that the cross-reactive T cell population with specificity for M1 and BMLF1 was functionally diverse, some cells producing multiple cytokines (MIP-1β, IFNγ, TNFα) and some cells producing only one or two cytokines (MIP-1β, or MIP-1β and IFNγ) to the same peptide stimulation. Furthermore, functional data also demonstrated a difference in the avidity between these cross-reactive T cells and each epitope that was dependent on the peptide used to culture the cells. It remained to be determined whether these observations could be explained by TCR structural diversity. Analyses of cross-reactive TCR structure and repertoire organization are an integral part of our research. The private specificity of each individual's antigen-specific TCR repertoire may promote the individual variability in disease severity associated with viral infections, such as EBV. It is possible that certain TCR structures correlate with enhanced immunopathology due to the signals they transmit upon cross-reactive stimulation. A strong signal may exacerbate an inflammatory response and lead to tissue damage, while a weak signal may result in an altered cytokine profile that could prevent efficient viral clearance and prolong a damaging inflammatory response. Once cross-reactive TCR structures are defined, the information can be used to track cross-reactive clones during the course of infection and between individuals. Cross-reactive TCR repertoire analyses will reveal the breadth and, therefore, the likelihood of a cross-

reactive T cell response. If multiple TCR structures can engage both epitopes, then there is an increased likelihood that a cross-reactive clone will be activated and function in response to infection. This is in contrast to the possible existence of a select few promiscuous clones, which may be relatively infrequent among individuals.

The Vβ17 family is a public component of an M1-specific TCR because of its pairing with multiple Vα families (Naumov et al., manuscript in preparation) and its dominance of the M1-specific repertoires of multiple individuals (Lawson et al., 2001; Lehner et al., 1995; Moss et al., 1991; Naumov et al., 2005; Naumov et al., 2003). Based on the crystal structure, we know that the CDR3β loop, and particularly its $arginine_{98}$ and $serine_{99}$ residues, is paramount in engaging the M1 epitope (Stewart-Jones et al., 2003). Less is known about the TCR requirements for interaction with the BMLF1 epitope, but it is interesting that, similar to Vβ17, the Vα15 family is a public component of a BMLF1-specific TCR because of its pairing with multiple Vβ families and its inclusion in the BMLF1-specific repertoires of multiple individuals (Annels et al., 2000; Cohen et al., 2002; Lim et al., 2000). The experiments presented here were aimed to determine the structure of this cross-reactive TCR and compare it to the known requirements for interaction with M1 and BMLF1. Population analyses provided a larger scope on the potential frequency of cross-reactive TCR structures within an individual's antigen-specific T cell population.

A. Experimental design

i. Defining cross-reactive and non-crossreactive T cell populations

Cross-reactive T cells have been identified by their ability to co-stain with M1-

and BMLF1-loaded tetramers, and this double-tetramer positive population was shown to produce MIP-1β, IFNγ, and TNFα following stimulation with either epitope (Chaper IV). However, subsets of functionally cross-reactive T cells also existed that only efficiently stained with one tetramer type. For instance, a subset (2%) of a CD8 T cell line grown in the presence of only M1 peptide was more efficient at staining with BMLF1-loaded tetramer, and we demonstrated that this BMLF1+ subset produced IFNγ (29%) in response to M1 peptide stimulation (Figure 22A). When present, we characterized the TCR repertoire of both cross-reactive T cells subsets, namely the M1/BMLF1 double-tetramer positive and the BMLF1 single-tetramer positive populations within M1-specific T cell lines. We defined non-crossreactive T cells as those cells which only stained with the tetramer loaded with the peptide used for culturing the cells, namely the M1 single-tetramer positive population within M1-specific T cell lines and the BMLF1 single-tetramer positive population within BMLF1-specific T cell lines. We either gated on these distinct, tetramer-defined, T cell subsets to assess TCR usage with Vβ-specific monoclonal antibodies or we sorted these distinct T cell subsets (Figure 22B, C) and used Vβ- and Vα-specific primers to assess TCR usage with a PCR-based technology, termed CDR3 spectratyping.

ii. The TCR repertoire of cultured T cell lines accurately reflects the antigen-specific TCR repertoire ex vivo

In order to fully investigate the organization and structural diversity of cross-reactive TCR repertoires, we first ensured that our culturing conditions supported the growth of all antigen-specific T cell clones. Since the population of BMLF1-specific

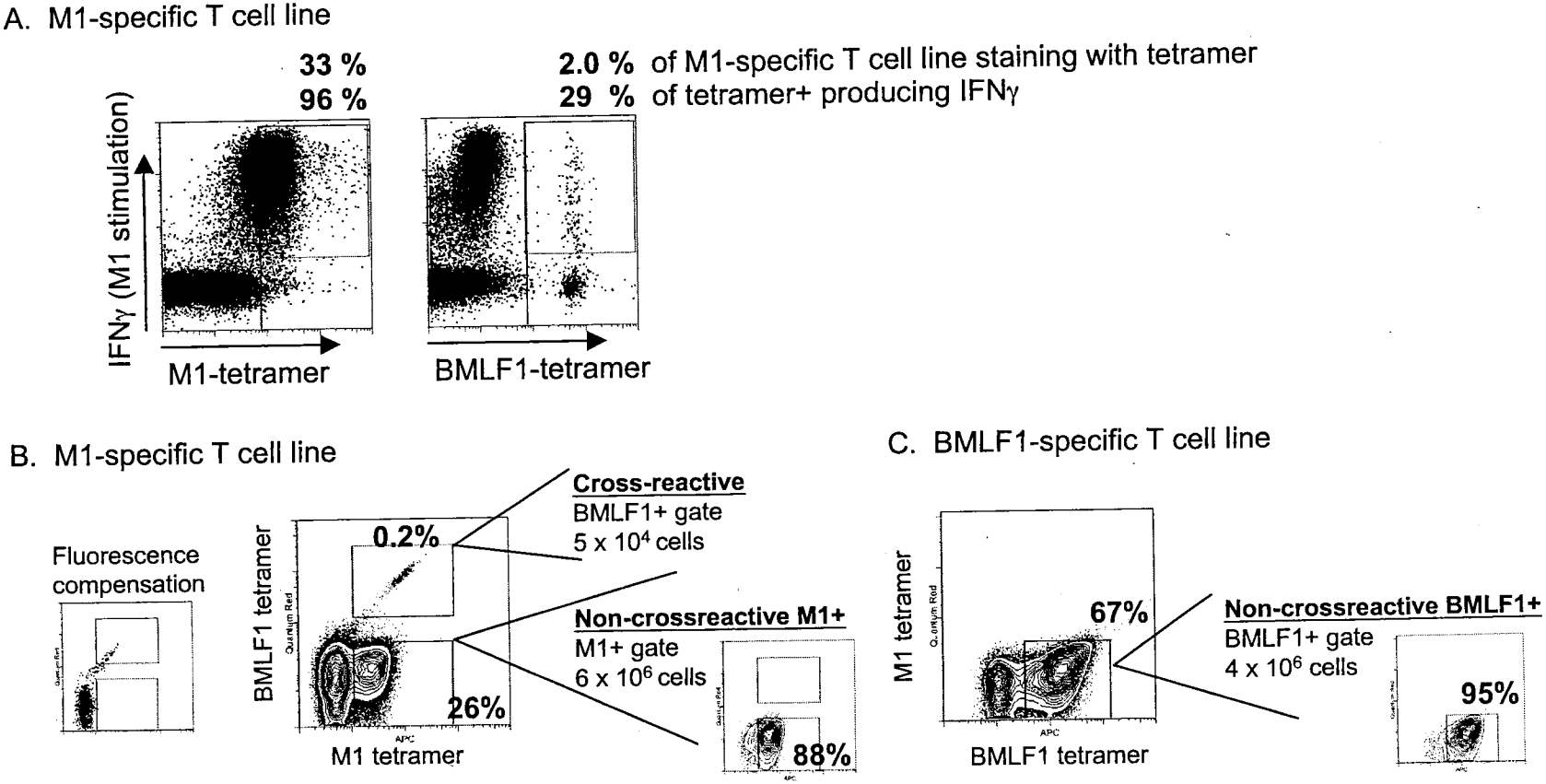


Figure 22. Cross-reactive and non-crossreactive T cell populations used for TCR analyses. T cell lines were derived from (A) IM patient E1101 or (B, C) healthy donor D-002. (A) The cross-reactive BMLF1+ subset of an M1-specific T cell line can produce IFNγ following M1 stimulation. (B) Two T cell subsets were isolated from an M1-specific line, cross-reactive BMLF1+ and non-crossreactive M1+. Note: The BMLF1+ subset is not thought to have any avidity for M1 tetramer and pulls off the axis as a result of insufficient compensation at the time of data collection. (C) One T cell subset was isolated from a BMLF1-specific line, non-crossreactive BMLF1+.

memory CD8 T cells in healthy donor D-002 was large enough to sort ex vivo, we compared the TCR Vβ usage of freshly isolated BMLF1-tetramer positive cells with those grown in the presence of BMLF1 peptide for 4 weeks and found the two repertoires were almost identical (Figure 23). Usage of the predominant Vβ14 (range: 35-63% of BMLF1+ cells) and Vβ16 (range: 18-27% of BMLF1+ cells) families was conserved. Our culturing conditions also effectively supported the growth of low frequency Vβ families, including Vβ17 (2-3%), Vβ18 (2-3%), and Vβ22 (5-6%). Furthermore, when we sub-cloned and sequenced their TCR CDR3β region, BMLF1+ sub-clones using both high and low frequency Vβ families were present in both ex vivo and in vitro TCR repertoires (Figure 23). TCR α-chain usage could not be assessed simultaneously and, therefore, we identified any sub-clone having a unique nucleotide sequence as a unique Vβ clonotype. The same dominant Vβ clonotype within each of the Vβ14, 17, 18, and 22 families was present ex vivo and in vitro. The dominant Vβ16+ clonotype ex vivo (ID: B16.2) became co-dominant in vitro with another Vβ16+ clonotype (ID: B16.1) that differed by only 1 of 8 aa residues comprising the CDR3β loop. These results suggested that the TCR repertoire of cultured T cell lines accurately reflects the antigen-specific repertoire present in peripheral blood.

These data were also in agreement with previous reports on BMLF1-specific TCR structure and BMLF1-specific TCR repertoire organization (Annels et al., 2000; Callan et al., 2000; Cohen et al., 2002; Lim et al., 2000). All four of the common Vβ families (Vβ2, 4, 16, 22) were represented in the BMLF1-specific TCR repertoire of healthy

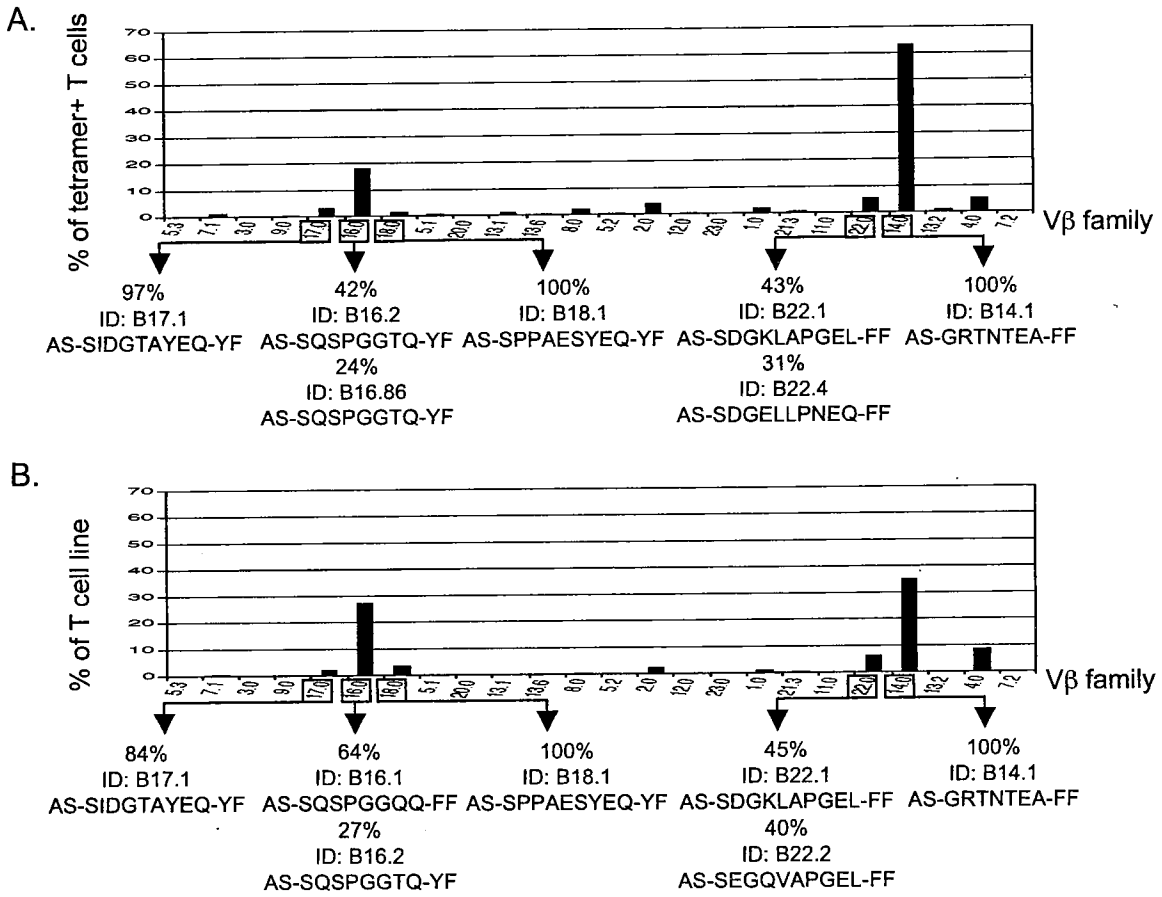


Figure 23. The Vβ repertoire of a cultured T cell line accurately reflects the antigen-specific Vβ repertoire ex vivo. CD8 T cells isolated from the PBMC of health donor D-002 were (A) immediately stained with BMLF1 tetramer or (B) cultured for 4 weeks in the presence of BMLF1 peptide and then stained with Vβ-specific antibodies. TCRs using the shaded Vβ families were sub-cloned. The ID of the predominant clonotype(s), the frequency among all sequences analyzed within that family, and the CDR3β aa sequence are shown. Nucleotide sequences of all clonotypes analyzed can be found in Table 7.

Table 7. Full sequences of the CDR3 regions of Vβ and Vα clonotypes.

Figure 23:

A. Donor D-002 ex vivo BMLF1+ sequences.

*Vβ	Jβ	Clonotype ID	Frequency	aa sequence CDR3β loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
14	1.1	B14.1	42	AS-GRTNTEA-FF	7	gccagcggtcggacgaacactgaagctttctt
		Total unique clonotypes:	*1*			
		Total sequences analyzed:	*42*			
16	2.5	B16.2	19	AS-SQSPGGTQ-YF	8	gccagcagccagagtccaggggggacccagtacttc
	2.5	B16.86	11	AS-SQSPGGTQ-YF	8	gccagcagccaaagtcccaggggggacccagtacttc
	2.5	B16.85	7	AS-SQSPGATQ-YF	8	gccagcagccaaagtccgggagcgacccagtacttc
	2.5	B16.4	3	AS-SQSPGGTQ-YF	8	gccagcagccagtccccggcgggacccagtacttc
	2.5	B16.5	1	AS-SQTPGGTQ-YF	8	gccagcagccagacccccgggggacccagtacttc
	2.5	B16.6	1	AS-SQSPGGTQ-YF	8	gccagcagccaatctctgggggacccagtacttc
	1.2	B16.3	1	AS-SQSPGGLE-TF	8	gccagcagccaatctccgggggcctcgagacctc
	2.1	B16.1	1	AS-SQSPGGQQ-FF	8	gccagcagccaatcccgggggtcagcagttcttc
	2.4	B16.7	1	AS-SQSPGGIQ-YF	8	gccagcagccaatcgcccggcgggattcagtacttc
		Total unique clonotypes:	*9*			
		Total sequences analyzed:	*45*			
17	2.7	B17.1	36	AS-SIDGTAYEQ-YF	9	gccagtagtatagacgggacagcctacgagcagtacttc
	2.1	B17.12	1	AS-SIDRGLINEQ-FF	10	gccagtagtatagatagaggacttatcaatgagcagttcttc
		Total unique clonotypes:	*2*			
		Total sequences analyzed:	*37*			
18	2.7	B18.1	38	AS-SPPAESYEQ-YF	9	gccagctcaccaccggccgagagctacgagcagtacttc
		Total unique clonotypes:	*1*			
		Total sequences analyzed:	*38*			
22	2.2	B22.1	17	AS-SDGKLAPGEL-FF	10	gccagcagcgacggaaaactagccccgggggagctgttttt
	2.1	B22.4	12	AS-SDGELLPNEQ-FF	10	gccagcagtgacggtgaactcctacccaatgagcagttcttt
	2.2	B22.2	7	AS-SEGQVAPGEL-FF	10	gccagcagtgaaggtcaggtggccccgggggagctgttttt
	2.2	B22.5	2	AS-SAGEVFPGEL-FF	10	gccagcagtgcagggaagttttccgggggagctgttttt
	2.2	B22.6	1	AS-SDGRVAPGEL-FF	10	gccagcagtgacggcagggtagctcccgggggagctgttttt
		Total unique clonotypes:	*5*			
		Total sequences analyzed:	*39*			

B. Donor D-002 in vitro BMLF1+ sequences.

*Vβ	Jβ	Clonotype ID	Frequency	aa sequence CDR3β loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
14	1.1	B14.1	10	AS-GRTNTEA-FF	7	gccagcggtcggacgaacactgaagctttctt
		Total unique clonotypes:	*1*			
		Total sequences analyzed:	*10*			
16	2.1	B16.1	7	AS-S QSPG GQQ-FF	8	gccagcagccaatcccgggggtcagcagttcttc
	2.5	B16.2	3	AS-S QSPG GTQ-YF	8	gccagcagccagagtccaggggggacccagtacttc
	1.2	B16.3	1	AS-S QSPG GLE-TF	8	gccagcagccaatctccgggggcctcgagacctc
		Total unique clonotypes:	*3*			
		Total sequences analyzed:	*11*			
17	2.7	B17.1	88	AS-SIDGTAYEQ-YF	9	gccagtagtatagacgggacagcctacgagcagtacttc
	2.1	B17.9	6	AS-SIMGGNYEQ-YF	9	gccagtagtataatgggggcaataatgagcagttcttc
	2.7	B17.2	1	AS-SIDGTAYEQ-YF	9	gccagtagtatcgacgggacagcctacgagcagtacttc
	2.7	B17.3	1	AS-SIDGTAYEQ-YF	9	gccagtagtatagacgggacagcctacgagcagtacttc
	2.7	B17.4	1	AS-SIDGTAYEQ-YF	9	gccagtagtatagacgggacggcctacgagcagtacttc
	2.7	B17.5	1	AS-SVDGTAYEQ-YF	9	gccagtagtgtagacgggacagcctacgagcagtacttc
	2.7	B17.6	1	AS-SIDGAAYEQ-YF	9	gccagtagtatagacgggacagcctacgagcagtacttc
	2.7	B17.8	1	AS-SINGTAYEQ-YF	9	gccagtagtataaacgggacagcctacgagcagtacttc
	1.3	B17.10	1	AS-SQSDSGYEQ-YF	9	gccagtagtccagtccgacttctggaaacaccatatatttt
	1.2	B17.11	1	AS-SSGTARYEQ-YF	9	gccagtagttccgggacagcccgctatggctacacacttc
	2.7	B17.12	1	AS-SISGTGYEQ-YF	9	gccagtagtatctccgggacagggcctacgagcagtacttc
	2.7	B17.7	2	AS-SIPRTGTAYEQ-YF	11	gccagtagtattcctcggacagggacagcctacgagcagtacttc
		Total unique clonotypes:	*12*			
		Total sequences analyzed:	*105*			
18	2.7	B18.1	14	AS-SPPAESYEQ-YF	9	gccagctcaccaccggccgagagctacgagcagtacttc
		Total unique clonotypes:	*1*			
		Total sequences analyzed:	*14*			
22	2.2	B22.1	9	AS-SDGKLAPGEL-FF	10	gccagcagcgacggaaaactagccccgggggagctgttttt
	2.2	B22.2	8	AS-SEGQVAPGEL-FF	10	gccagcagtgaaggtcaggtggccccgggggagctgttttt
	1.2	B22.3	2	AS-NEGAGFNYGY-TF	10	gccagcaacgaaggggccggatttaactatggctacacctc
	2.1	B22.4	1	AS-SDGELLPNEQ-FF	10	gccagcagtgacggtgaactcctacccaatgagcagttcttc
		Total unique clonotypes:	*4*			
		Total sequences analyzed:	*20*			

Figure 27:

A. Donor D-002 cross-reactive sequences in an M1-specific T cell line.

*Vβ	Jβ	Clonotype ID	Frequency	aa sequence CDR3β loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
14	1.1	B14.1	16	AS-GRTNTEA-FF	7	gccagcggtcggacgaacactgaagctttctt
	2.1	14.6	4	AS-SSGSSGYYNEQ-FF	11	gccagcagttccgggtcctcgggtactacaatgagcagttcttc
	2.1	14.15	1	AS-SLGTSGSYNEQ-FF	11	gccagcagttaggtactagcgcgggagttacaatgagcagttcttc
	1.1	14.24	1	AS-GRTNIEA-FF	7	gccagcggtcggacgaacatgaagctttctt
	2.1	14.28	1	AS-SFGTSPYNEQ-FF	10	gccagcagttcgggactagtccctacaatgagcagttcttc
		Total unique clonotypes:	*5*			
		Total sequences analyzed:	*23*			
17	2.7	17.3	4	AS-SIRSSYEQ-YF	8	gccagtagtatcaaggagctcctacgagcagtacttc
		17.27	3	AS-SIDGTAYEQ-YF	9	gccagtagtatagacgggacagcctacgagcagtacttc
		17.13	2	AS-SIRSSYEQ-YF	8	gccagtagtatacggtcgtcttacgagcagtacttc
		17.16	2	AS-SVRSSYEQ-YF	8	gccagtagtgttagaagctcctacgagcagtacttc
		17.26	2	AS-STRSSYEQ-YF	8	gccagtagtacgcgaagctcctacgagcagtacttc
		17.21	2	AS-SMRSAYEQ-YF	8	gccagtagtatgcgggagcgcctacgagcagtacttc
		17.6	1	AS-SIRSSYEQ-YF	8	gccagtagtatacgaagctcctacgagcagtacttc
		17.12	1	AS-S IRSS YEQ-YF	8	gccagtagtatccgaagctcctacgagcagtacttc
		17.20	1	AS-SIRSSYEQ-YF	8	gccagtagtattcgaagtagttacgagcagtacttc
		17.5	1	AS-SIRSSYEQ-YF	8	gccagtagtataaggttcttcctacgagcagtacttc
		17.28	1	AS-SIRSSYEQ-YF	8	gccagtagtattcggagttcctacgagcagtacttc
		17.32	1	AS-SIRSSYEQ-YF	8	gccagtagtattcgttcctcctacgagcagtacttc
		17.36	1	AS-SIRSSYEQ-YF	8	gccagtagtattagaagctcctacgagcagtacttc
		17.39	1	AS-SIRSSYEQ-YF	8	gccagtagtattcggtcatcctacgagcagtacttc
		17.49	1	AS-SIRSSYEQ-YF	8	gccagtagtatacgtagctcctacgagcagtacttc
		17.57	1	AS-SIRSSYEQ-YF	8	gccagtagtatcaggagttcctacgagcagtacttc
		17.62	1	AS-SIRSSYEQ-YF	8	gccagtagtatcaggagctcctacgagcagtacttc
		17.8	1	AS-SVRSSYEQ-YF	8	gccagtagtgtcagaagctcctacgagcagtacttc
		17.30	1	AS-SVRSSYEQ-YF	8	gccagtagtgtcaggagctcctacgagcagtacttc
		17.37	1	AS-SVRSSYEQ-XF	8	gccagtagtgtacggagctcctacgagcagtanttc
		17.38	1	AS-SVRSSYEQ-YF	8	gccagtagtgttaggtcctcctacgagcagtacttc
		17.43	1	AS-SVRSSYEQ-YF	8	gccagtagtgtacgtagctcctacgagcagtacttc
		17.54	1	AS-STRSSYEQ-YF	8	gccagtagtacacgatcttcctacgagcagtacttc
		17.64	1	AS-SLRSSYEQ-YF	8	gccagtagttgagaagctcctacgagcagtacttc
		17.11	1	AS-SMRSSYEQ-YF	8	gccagtagtatgaggagctcctacgagcagtacttc
		17.19	1	AS-SSRSSYEQ-YF	8	gccagtagtagtcggagctcctacgagcagtacttc
		17.56	1	AS-SSRSAYEQ-YF	8	gccagtagttctcgatccgcctacgagcagtacttc
		17.40	1	AS-SMRAAYEQ-YF	8	gccagtagtatgcgggcagcctacgagcagtacttc

(Table 7 continued)

2.3	17.2	2	AS-SKRSTDTQ-YF	8	gccagtagcaaaaggagcacagatacgcagtatttt
	17.15	2	AS-SQRSTDTQ-YF	8	gccagtagtcagagaagcacagatacgcagtatttt
	17.10	1	AS-SPRSTDTQ-YF	8	gccagtagtccccggagcacagatacgcagtatttt
	17.29	1	AS-SPRSTDTQ-YF	8	gccagtagtccccggtccacagatacgcagtatttt
	17.24	1	AG-SGRSTDTQ-YF	8	gccggtagcggacggagcacagatacgcagtatttt
	17.55	1	AS-SGRSTDTQ-YF	8	gccagtagcgggagaagcacagatacgcagtatttt
	17.50	1	AS-STRSSDTQ-YF	8	gccagtagtacccgatcgtcagatacgcagtatttt
	17.60	1	AS-SIRSADTQ-YF	8	gccagtagtataaggtcggcggatacgcagtatttt
	17.33	1	AS-SIRPADTQ-YF	8	gccagtagtataaggccggccggatacgcagtatttt
2.1	17.41	2	AS-SALAGPTYNEQ-FF	11	gccagtccgcgctagcgggaccaacctacaatgagcagttcttc
	17.31	1	AS-SIGTGEQ-FF	7	gccagtagtatcggtacaggggagcagttcttc
	17.65	1	AS-S IETTSGSSYNEQ-FF	13	gccagtagtatcgagacgactagcggaagctcctacaatgagcagttcttc
1.2	17.48	2	AS-S IGIYGY-TF	7	gccagtagtatcgggatctatggctacacctttcggttcg
	17.52	1	AS-SSGSYGY-TF	7	gccagtagttcgggcagttatggctacacctttcggttcg
	Total unique clonotypes:	*42*			
	Total sequences analyzed:	*55*			

B. Donor D-002 non-crossreactive M1+ sequences in an M1-specific T cell line.

*Vβ	Jβ	Clonotype ID	Frequency	aa sequence CDR3β loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
17	2.7	43.27	6	AS-SMRSSYEQ-YF	8	gccagtagtatgaggagttcctacgagctgtacttc
		17.27	5	AS-SIRSSYEQ-YF	8	gccagtagtataagaagctcctacgagcagtacttc
		23.27	3	AS-SIRSSYEQ-YF	8	gccagtagtatccgttcgtcctacgagcagtacttc
		28.27	3	AS-SIRSSYEQ-YF	8	gccagtagtatccaggagctcctacgagcagtacttc
		6.27	2	AS-SIRSSYEQ-YF	8	gccagtagtatccgaagctcctacgagcagtacttc
		25.27	2	AS-SIRSSYEQ-YF	8	gccagtagtatacgtagctcctacgagcagtacttc
		36.27	2	AS-STRSSYEQ-YF	8	gccagtagtaccaggagctcctacgagcagtacttc
		51.27	2	AS-SVRSSYEQ-YF	8	gccagtagtgtcagaagctcctacgagcagtacttc
		72.27	2	AS-ALRSSYEQ-YF	8	gccagtagtttgagaagctcctacgagcagtacttc
		73.27	2	AS-SIRAAYEQ-YF	8	gccagtagtaccgggcagcctacgagcagtacttc
		84.27	2	AS-SIRSGYEQ-YF	8	gccagtagtatttcgatcagggtacgagcagtacttc
		86.27	2	AS-SFRSAYEQ-YF	8	gccagtcgcttaggagtgcctacgagcagtacttc
		1.27	1	AS-SIRSSYEQ-YF	8	gccagtagtattaggagctcctacgagcagtacttc
		3.27	1	AS-SIRSSYEQ-YF	8	gccagtagtatcagaagctcctacgagcagtacttc
		19.27	1	AS-SIRSSYEQ-YF	8	gccagtagtattcggagctcctacgagcagtacttc
		22.27	1	AS-SIRSSYEQ-YF	8	gccagtagtattcgttcgtcctacgagcagtacttc
		29.27	1	AS-SIRSSYEQ-YF	8	gccagtagtatacggagctcctacgagcagtacttc
		35.27	1	AS-STRSSYEQ-YF	8	gccagtagtacgcgaagctcctacgagcagtacttc
		38.27	1	AS-STRSSYEQ-YF	8	gccagtagtaccaggtcttcctacgagcagtacttc
		39.27	1	AS-STRSSYER-YF	8	gccagtagtactaggagctcctacgagcagtacttc
		40.27	1	AS-STRSSYER-YF	8	gccagtagtacacgatcttcctacgagcagtacttc
		41.27	1	AS-STRSSYER-YF	8	gccagtagtaccaggagctcctacgagcagtacttc
		49.27	1	AS-SVRSSYEQ-YF	8	gccagtagtgttcggagctcctacgagcagtacttc
		59.27	1	AS-SSRSAYEQ-YF	8	gccagtagttccaggtccgcctacgagcagtacttc
		60.27	1	AS-SSRSAYEQ-YF	8	gccagtagttctcgatccgcctacgagcagtacttc
		85.27	1	AS-SMRSAYEQ-YF	8	gccagtagtatgcggagcgcctacgagcagtacttc
		91.27	1	AS-SIRSAYEQ-YF	8	gccagtagtatccgcagtgcctacgagcagtacttc
	2.3	1.23	3	AS-SGRSTDTQ-YF	8	gccagtagcgggagaagcacagatacgcagtatttt
		5.23	1	AS-SPRSGDTQ-YF	8	gccagttcccgcgcagtggggatacgcagtatttt
		10.23	1	AS-SSRSTDTQ-YF	8	gccagtagttcaagatccacagatacgcagtatttt
		16.23	1	AS-SQRSTDTQ-YF	8	gccagtagtccgagaagcacagatacgcagtatttt
		18.23	1	AS-SQRSTDTQ-YF	8	gccagttccgggaggagcacagatacgcagtatttt
		19.23	1	AS-SQRSTDTQ-YF	8	gccagtagcagagaagcacagatacgcagtatttt
		25.23	1	AS-STRSSDTQ-YF	8	gccagtagtacccgatcgtcagatacgcagtatttt
		30.23	1	AS-SIRSTDTQ-YF	8	gccagtagtataagaagcacagatacgcagtatttt
		32.23	1	AS-STRSADTQ-YF	8	gccagtagtactagaagcgcagatacgcagtatttt
		33.23	1	AS-SMRTGDTQ-YF	8	gccagtagtatgcggaccggtgatacccagtatttt
	2.1	1.21	1	AT-SGRAGVEQ-FF	8	gccacctccggccgggccgggggt tgagcagttcttc
		7.21	1	AS-SYRSSNEQ-FF	8	gccagtagttcaggtcgtccaatgagcagttcttc
		2.21	2	AS-SIGTGEQ-FF	7	gccagtagtatcggtacaggggagcagttcttc
		Total unique clonotypes:	*40*			
		Total sequences analyzed:	*64*			

Figure 28:

A. Donor D-002 non-crossreactive BMLF1+ sequences in an BMLF1-specific T cell line.

*Vα	Jα	Clonotype ID	Frequency	aa sequence CDR3α loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
15	45	B1	38	AE-RKGGADGL-TF	8	gcagagagaaaaggaggtgctgacggactcaccttt
	3	B2	5	AE-YSSASKI-IF	7	gcagagtacagcagtgcttccaagataatctttt
	37	B3	2	AE-STGKL-IF	5	gcagagtcaacaggcaaactaatctttt
	41	B47	2	AE-SKGAL-NF	5	gcagagagtaaaggcgcactcaactc
	15	B16	1	AE-SEGTAP-IF	6	gcagagagtgaaggaactgctccgatcttt
	3	B31	1	AE-YSSAPKI-IF	7	gcagagtacagcagtgctcccaagataatctttt
	5	B38	1	AE-SRGAL-TF	5	gcagagagtcgaggagcacttactttt
		Total unique clonotypes:	*7*			
		Total sequences analyzed:	*50*			

B. Donor D-002 crossreactive sequences in an M1-specific T cell line.

*Vα	Jα	Clonotype ID	Frequency	aa sequence CDR3α loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
15	45	B1	35	AE-RKGGADGL-TF	8	gcagagagaaaaggaggtgctgacggactcaccttt
	37	X5	5	AE-STGKL-IF	5	gcagagtcgacaggcaaactaatctttt
	13	X3	3	AE-LGYQKV-TF	6	gcagagttgggttaccagaaagttacctttt
	15	X12	3	AE-SEGTAL-IF	6	gcagagagtgaaggaactgctctgatcttt
	11	X45	2	AE-DRDSTL-TF	6	gcagagggacgggacagcaccctcaccttt
	15	B16	1	AE-SEGTAP-IF	6	gcagagagtgaaggaactgctccgatcttt
	45	X23	1	AE-RKGGANGL-TF	8	gcagagagaaaaggaggtgctgacggactcaccttt
		Total unique clonotypes:	*7*			
		Total sequences analyzed:	*50*			

C. Donor D-002 non-crossreactive M1+ sequences in an M1-specific T cell line.

*Vα	Jα	Clonotype ID	Frequency	aa sequence CDR3α loop	**Length (aa)	Nucleotide sequence of V/N/D/N/J region
15	23	M2	19	AE-DNQGGKL-IF	7	gcagaggataacaggggaggaaagcttatctttc
	8	M1	6	AE-SPSLNTGFQKL-VF	11	gcagagagccctcccgaacacaggctttcagaaacttgtatttt
	43	M8	3	AE-SNNDM-RF	5	gcagagagtaacaatgacatgcgctttt
	42	M70	2	AE-PGSQGNL-IF	7	gcagagccgggaagccaaggaaatctcatctttt
	42	M25	1	AE-PGSQGNL-VF	7	gcagagccgggaagccaaggaaatctcgtctttt
		Total unique clonotypes:	*5*			
		Total sequences analyzed:	*31*			

Each unique clonotype is distinguished by its unique nucleotide sequence. * TCR variable (V) region nomenclature based on Arden et al., ** CDR3 loop length according to Chothia et al. shown flanked by two framework regions.

donor D-002 (Figure 23). Furthermore, we confirmed the observation that a given Vβ family is often comprised of only 1-2 unique clonotypes (Callan et al., 2000). For donor D-002, as few as 2 clonotypes accounted for greater than 60% of each Vβ population investigated (Figure 23). By comparison, the clonotypes within each Vβ family varied in their length and the aa sequence of their CDR3β loop, the most common residue among them being glycine (detected in the clonotypes of 4 of 5 Vβ families). The dominant Vβ clonotypes detected in our study used the conserved Jβ family and/or CDR3β motif associated with those Vβ families for which public specificities have previously been reported (Table 2). Vβ16+ clonotypes used the common CDR3β motif AS-SQSPGGTQ-YF and had less restrictions on their Jβ usage, while Vβ22+ clonotypes were more restricted by the usage of Jβ2.1 or Jβ2.2 than the aa sequence of their CDR3β loop, AS-S*G*V*PGEL-FF (Figure 23) (Lim et al., 2000). In addition, these data revealed novel observations on the BMLF1-specific TCR repertoire, such as the usage of Vβ families 14, 17 and 18. All three Vβ families were comprised of one dominant clonotype with a CDR3β loop sequence that may be conserved in other donors with BMLF1-specific repertoires that include these particular Vβ families (Figure 23). Overall, the 5 Vβ families analyzed here (Vβ14, 16, 17, 18, 22) represented over 80% of the BMLF1-specific repertoire of donor D-002, and the total number of unique Vβ clonotypes identified out of the total number of sequences analyzed was as few as 18 out of 201 isolated ex vivo and 21 out of 160 isolated in vitro (Table 7), reaffirming that the BMLF1-specific TCR repertoire is quite oligoclonal in comparison to the (IV)M1-

specific TCR repertoire, where as many as 141 unique clonotypes were previously identified from 501 total sequences analyzed within the Vβ17 family alone (Naumov et al., 1998; Naumov et al., 2003).

B. Characterization of cross-reactive TCR structure and repertoire organization

We used tetramers and Vβ-specific monoclonal antibodies to first describe the cross-reactive TCR repertoires of 2 healthy donors and 4 patients with acute EBV infection. Among these 6 individuals, we determined that each cross-reactive repertoire contained 1 or more of 10 different Vβ families, including Vβ1, 2, 4, 5.1, 7.2, 14, 16, 17, 21.3 and 22 (Figures 24, 25). Thus, a wide range of TCR structures appeared capable of recognizing the two dissimilar epitopes M1 and BMLF1. We found no discernable difference in the breadth of the cross-reactive repertoires of healthy donors compared to patients with acute EBV infection. While we were unable to screen for all of the known Vβ families due to the lack of the appropriate monoclonal antibodies, the cross-reactive repertoire of at least one individual used as few as 1 Vβ family (patient E1178, Vβ1) and the cross-reactive repertoire of another individual used as many as 6 different Vβ families (patient E1109, Vβ4, 5.1, 7.2, 14, 17 and 21.3). The breadth of the cross-reactive Vβ repertoire was most extensive (≥5 Vβ families) in the two patients, E1101 and E1109, having two distinct, tetramer-defined, sub-populations of cross-reactive cells (Figures 24, 25). The most common Vβ family detected among the cross-reactive repertoires of all individuals was Vβ4, used by 4 out of 6 individuals.

We have just begun to assess the Vα usage of these same cross-reactive T cell

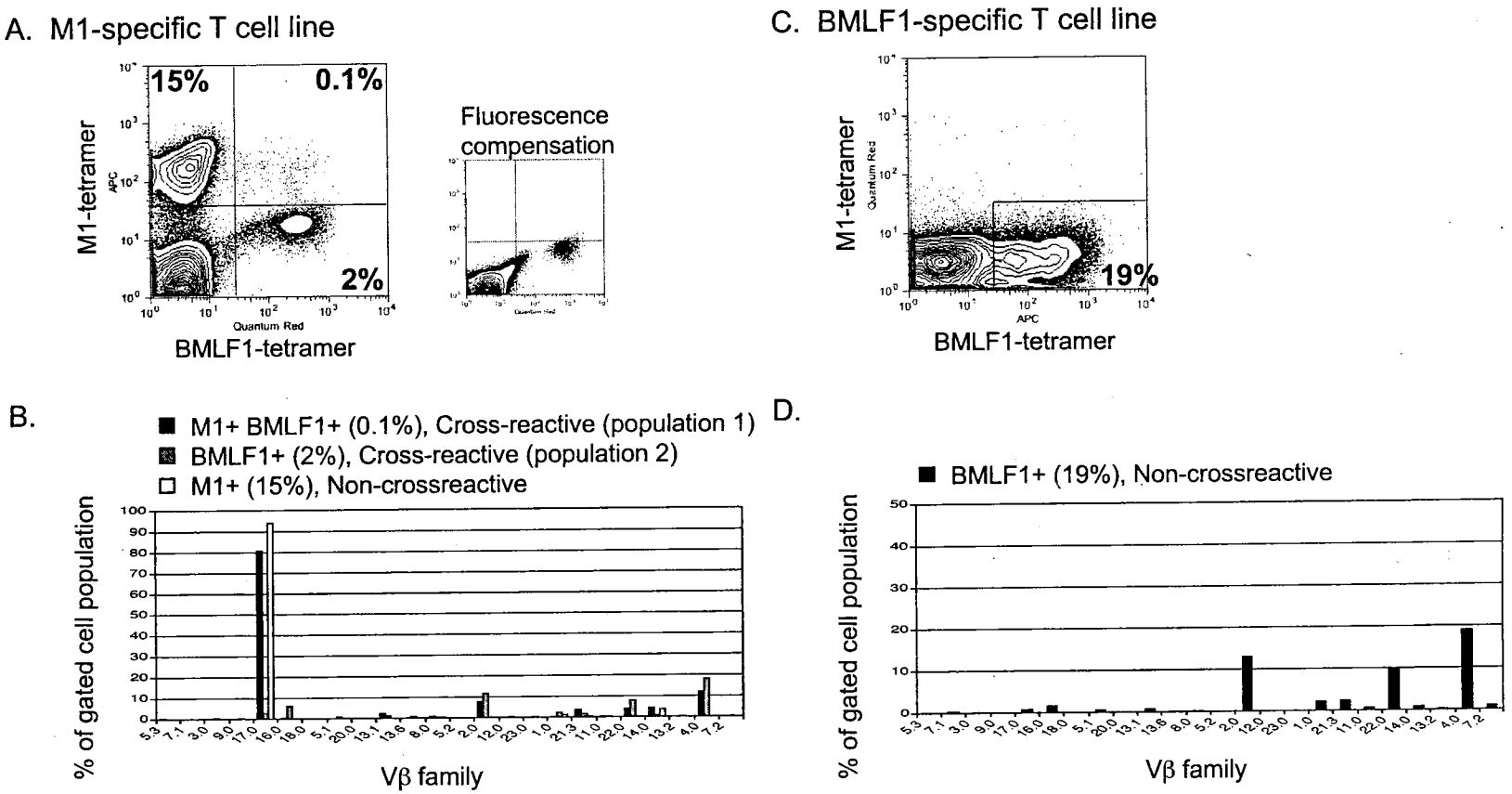


Figure 24. Each tetramer-defined subset of a T cell line has a distinct Vβ repertoire. CD8 T cells derived from IM patient E1101 were cultured with (A) M1 or (C) BMLF1 peptide for 3 weeks and then co-stained with M1 and BMLF1 tetramers. Note: the BMLF1+ subset in (A) is not thought to have any avidity for M1 tetramer and pulls off the axis as a result of insufficient compensation at the time of data collection. Tetramer stains within the (B) M1-specific or (D) BMLF1-specific T cell lines were used to separately gate on different subsets and view the % that stained with Vβ-specific antibodies.

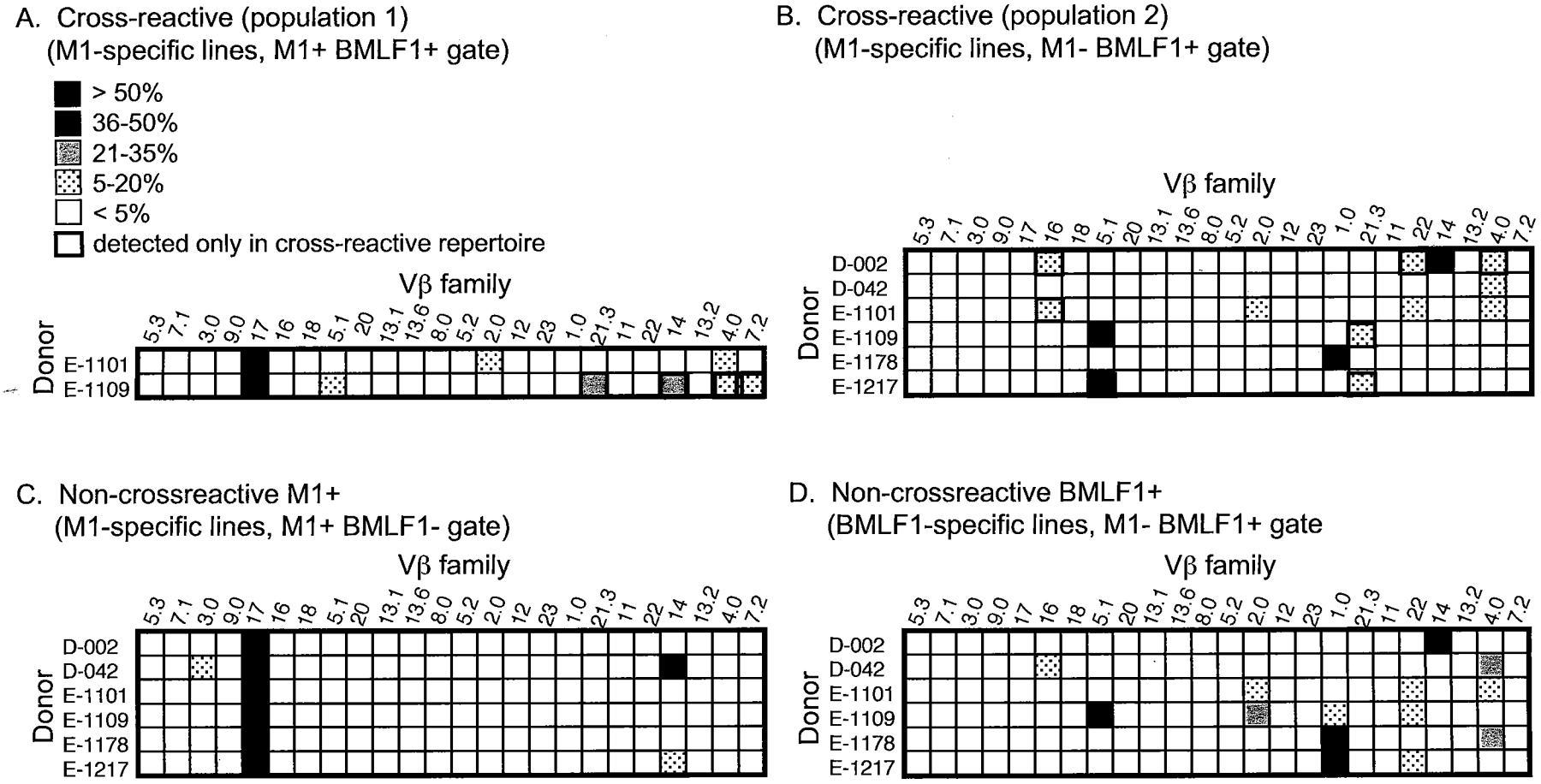


Figure 25. The Vβ repertoire of cross-reactive T cells is often comprised of multiple Vβ families and is unique to each individual. (A-C) M1- and (D) BMLF1-specific T cell lines were derived from 2 healthy donors and 4 IM patients and were cultured for 3-4 weeks. (A-C) The M1-specific lines were separated into 3 distinct subsets based on their M1 and BMLF1 tetramer staining profile and the % staining with each Vβ-specific antibody is shown. (D) The Vβ repertoire of the BMLF1 tetramer+ subset of each BMLF1-specific T cell line.

subsets. We isolated the BMLF1+ cross-reactive T cell subset within the M1-specific line derived from healthy donor D-002 (Figure 22B) and, in a screen that included primers specific for 24 different Vα families, this cross-reactive repertoire included at least 9 different Vα families (Vα1b, 2, 6, 10, 11, 15, 20, 23 and 24) (Figure 26A). Thus, as was observed during analyses of Vβ usage, a wide range of Vα families can potentially mediate TCR interaction with these two dissimilar epitopes.

C. Distinguishing features of the cross-reactive TCR repertoire

i. Population analyses of TCR β-chain usage

Given the wide range of possible cross-reactive TCR structures and the individual variability previously documented for both the Vβ composition of BMLF1-specific repertoires and the clonal composition of Vβ17+ M1-specific repertoires, we compared the cross-reactive and non-crossreactive repertoires of each individual separately for insight on the organization behind each cross-reactive repertoire. Despite representing a subset of the M1-specific and BMLF1-specific T cell populations, we observed several aspects of the cross-reactive TCR repertoire that made it unique. For instance, although the same Vβ families were often used by non-cross-reactive and cross-reactive T cell subsets, the proportions of those Vβ families were always altered in the cross-reactive repertoires. In the case of IM patient E1101, a cross-reactive population that co-stained with both M1- and BMLF1-loaded tetramers predominantly used Vβ17, as did this patient's non-cross-reactive M1-specific population, but that particular cross-reactive repertoire also included low frequency Vβ families (Vβ2 and 4) which were not components of the non-crossreactive M1-specific repertoire (Figure 24). In addition, the

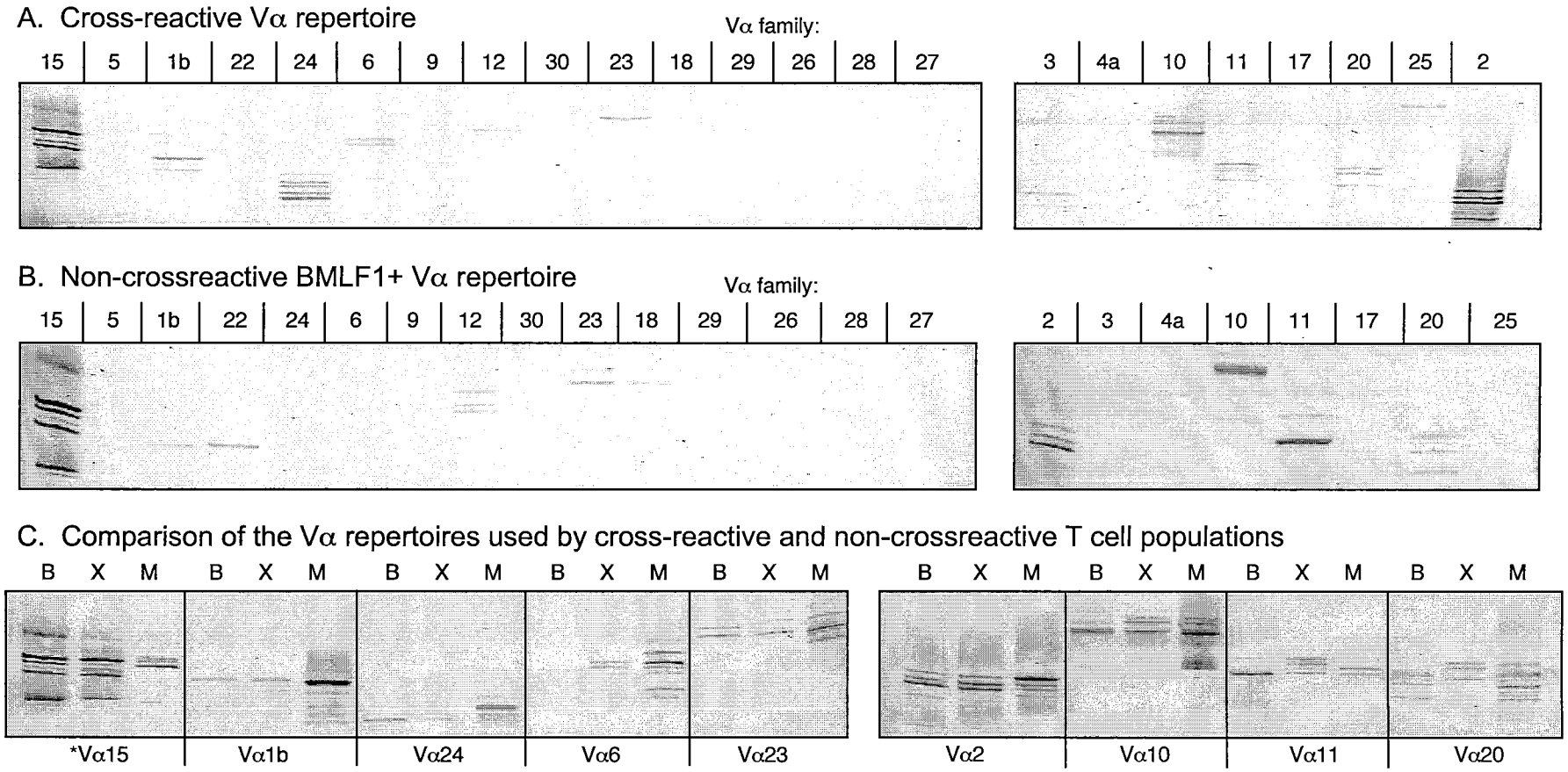


Figure 26. The Vα repertoire of cross-reactive T cells is comprised of multiple Vα families and is distinct from that of non-crossreactive M1+ or BMLF1+ T cells. CDR3α spectratyping analyses on the (A) cross-reactive, (B) non-crossreactive BMLF1+, or (C) all three T cell subsets outlined in Figure 22B-C (B: non-crossreactive BMLF1+, X: cross-reactive, M: non-crossreactive M1+ T cell subset). * Clonotypes comprising the Vα15 family were later sequenced.

cross-reactive subset that bound only BMLF1-loaded tetramer, within the M1-specific T cell line derived from this same patient, used three Vβ families common to the non-crossreactive BMLF1-specific repertoire (Vβ2, 4 and 22), but remained distinct by using an increased frequency of Vβ16 (6%) which was barely detectable in the non-crossreactive BMLF1-specific repertoire (Figure 24).

In addition to using altered proportions of the same Vβ families, selective gating on cross-reactive T cell subsets appeared to enhance the detection of different Vβ families used more frequently by cross-reactive than non-cross-reactive T cells. The cross-reactive repertoires of 4 out of 6 individuals investigated included 1 or more Vβ families not detected within either of that individual's non-crossreactive repertoires (Figure 25A, B). In some cases, the Vβ families strictly detected in the cross-reactive repertoire included Vβ families common to other individuals, such as Vβ4, 16 and 22. In other cases, the Vβ families strictly detected in the cross-reactive repertoire were rare even among the repertoires of other individuals, such as Vβ5.1, 7.2 and 21.3. The most extreme example of this was observed upon the repertoire analysis of patient E1217, whose cross-reactive repertoire consisted entirely of two Vβ families (Vβ5.1 and Vβ21.3) that were not detectable in either non-crossreactive repertoire.

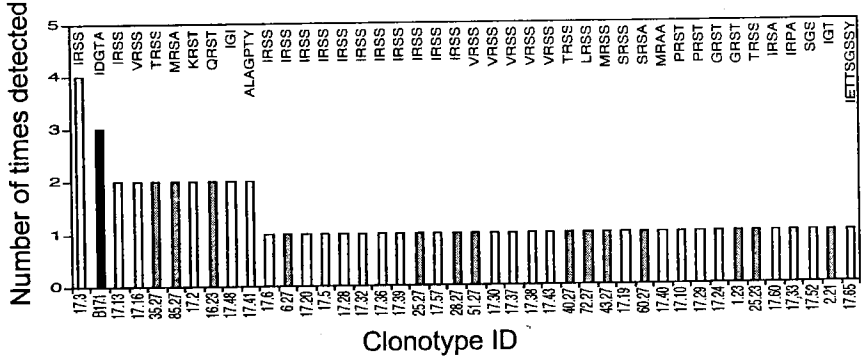
There were also differences on a clonal level that made the cross-reactive repertoire distinct from either non-crossreactive repertoire. When we sub-cloned and sequenced Vβ14+ T cells derived from healthy donor D-002, representing the dominant Vβ family of both the cross-reactive and non-crossreactive BMLF1-specific repertoires,

we detected an increased number of Vβ14+ clonotypes in the cross-reactive population (5 unique clonotypes out of 23 total sequences) compared to the restricted clonal composition of the non-crossreactive Vβ14+ BMLF1-specific population (1 unique clonotype out of 10 total sequences) (Figures 23 and 27A). Three of the four novel Vβ14+ cross-reactive clonotypes used an alternative Jβ family (Jβ2.1) and a longer CDR3β loop (10-11aa length). Interestingly, we had previously sub-cloned and sequenced the small Vβ14+ subset (2%) within the non-crossreactive M1-specific population and detected only 1 unique clonotype out of 82 total sequences (Jβ2.1, 9aa length, AS-SSRGHGNEQ-FF), which had a surprisingly similar structure to the newly identified cross-reactive Vβ14+ clonotypes (Jβ2.1, 10-11aa length, CDR3β motif: AS-S*GTSG*YNEQ-FF) (Figure 27A) (Naumov et al., 2005). The longer CDR3β regions were extended by multiple glycine or serine residues at the point of gene recombination. Both amino acid residues have uncharged, non-bulky side chains that may give the CDR3β loop flexibility and potentially allow these TCRs to adapt to an alternative ligand (Abergel and Claverie, 1991; Bentley and Mariuzza, 1996). Similarly, we sub-cloned and sequenced several novel Vβ17+ cross-reactive clonotypes, the dominant Vβ family of the non-crossreactive M1-specific repertoire. The majority of the cross-reactive Vβ17+ repertoire (64% of the unique clonotypes) was comprised of new clonotypes that were not previously detected in either non-crossreactive repertoire and, once again, included clonotypes with extended CDR3β regions (Figure 27B).

A. Cross-reactive Vβ14+ clonotypes (n = 23)

Freq	ID	Jβ	AA seq of CDR3β loop	Length
16	**B14.1**	**1.1**	**AS-GRTNTEA-FF**	7
4	14.6	2.1	AS-SSGSSGYYNEQ-FF	11
1	14.15	2.1	AS-SLGTSGSYNEQ-FF	11
1	14.24	1.1	AS-GRTNIEA-FF	7
1	14.28	2.1	AS-SFGTSPYNEQ-FF	10

B. Cross-reactive Vβ17+ clonotypes (n = 55)



C. Non-crossreactive M1+ Vβ17+ clonotypes (n = 64)

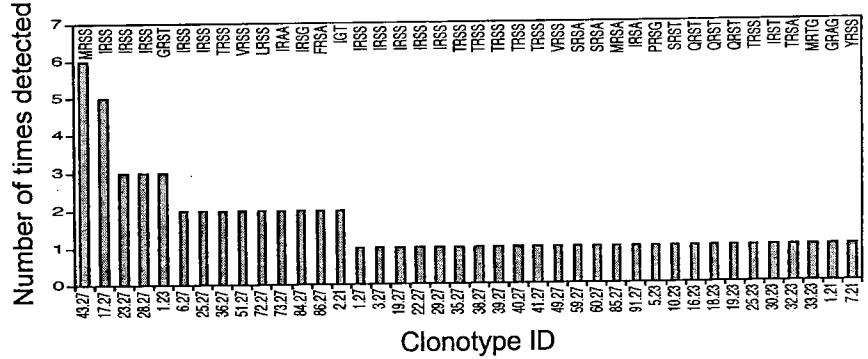


Figure 27. The Vβ-specific repertoires of a cross-reactive T cell population are comprised of multiple clonotypes that use a combination of M1- and BMLF1-specific TCR elements. (A) The CDR3β sequences of cross-reactive Vβ14+ clonotypes. Based on a unique nucleotide sequence, clonotypes were assigned an ID and the number of times that clonotype was detected among the total sequences analyzed (frequency) is shown. The length of the CDR3β region is according to Chothia et al., shown supported by two flanking framework regions. Bold indicates that the clonotype was previously detected within a BMLF1-specific population (Figure 23). (B, C) The CDR3β sequences of Vβ17+ clonotypes found within the (B) crossreactive or (C) non-crossreactive M1-specific T cell subsets, arranged in a power law-like distribution based on their frequency. The length of the CDR3β regions has been abbreviated. (white bars: unique to cross-reactive subset, grey bars: detected within the non-crossreactive M1+ subset, black bars: detected within the non-crossreactive BMLF1+ subset) Nucleotide sequences of all clonotypes analyzed can be found in Table 7.

ii. Population analyses of TCR α-chain usage

Vα repertoire analyses of T cell populations derived from healthy donor D-002 were performed using a PCR-based technique and, therefore, did not reveal the relative frequencies of Vα family usage. However, we did compare the clonal composition of several Vα families used by these T cell populations by running the Vα-specific CDR3 spectratyping reactions of each population on the same gel (Figure 26C). This technique revealed differences between the repertoires of these populations in the lengths of the CDR3α regions, an indication of clonal differences within a given Vα family. This was evident among Vα11+ cross-reactive T cells which clearly expressed a longer CDR3α region than either non-crossreactive M1-specific or non-crossreactive BMLF1-specific T cells (Figure 26C). Similarly, the CDR3α spectratyping pattern of Vα20+ cross-reactive T cells also appeared distinct from that of either non-crossreactive population. Unique TCR α-chain features of the cross-reactive repertoire were also observed at the clonal level. Despite having some similarities with the BMLF1-specific Vα15+ repertoire, the cross-reactive Vα15+ repertoire included clonotypes that expressed unique Jα families (Jα11 and Jα13) and CDR3α loops 6aa long, a length which was more frequent in the cross-reactive repertoire (18% of sequences) than the non-crossreactive BMLF1-specific repertoire (2% of sequences) (Figure 28A, B). Furthermore, there were no similarities between the cross-reactive and non-crossreactive M1-specific Vα15+ repertoires (Figure 28C).

A. Cross-reactive (X) Vα15+ clonotypes (n= 50):

Freq	ID	Jα	AA seq of CDR3α loop	Length
70%	**B1**	**45**	**AE-RKGGADGL-TF**	**8**
10%	X5	37	AE-STGKL-IF	5
6%	X3	13	AE-LGYQKV-TF	6
6%	X12	15	AE-SEGTAL-IF	6
4%	X45	11	AE-DRDSTL-TF	6
2%	**B16**	**15**	**AE-SEGTAP-IF**	**6**
2%	X23	45	AE-RKGGANGL-TF	8

B. Non-crossreactive BMLF1+ (B) Vα15+ clonotypes (n=50):

Freq	ID	Jα	AA seq of CDR3α loop	Length
76%	B1	45	AE-RKGGADGL-TF	8
10%	B2	3	AE-YSSASKI-IF	7
4%	B3	37	AE-STGKL-IF	5
4%	B47	41	AE-SKGAL-NF	5
2%	B16	15	AE-SEGTAP-IF	6
2%	B31	3	AE-YSSAPKI-IF	7
2%	B38	5	AE-SRGAL-TF	5

C. Non-crossreactive M1+ (M) Vα15+ clonotypes (n=31):

Freq	ID	Jα	AA seq of CDR3α loop	Length
61%	M2	23	AE-DNQGGKL-IF	7
19%	M1	8	AE-SPSLNTGFQKL-VF	11
10%	M8	43	AE-SNNDM-RF	5
7%	M70	42	AE-PGSQGNL-IF	7
3%	M25	42	AE-PGSQGNL-VF	7

Figure 28. The Vα15+ repertoire of cross-reactive T cells is comprised of clonotypes that use BMLF1-specific and unique TCR elements. The CDR3α sequences of Vα15+ clonotypes within the (A) cross-reactive, (B) non-crossreactive BMLF1+, or (C) non-crossreactive M1+ T cell subsets of healthy donor D-002. Based on a unique nucleotide sequence, clonotypes were assigned an ID and the frequency (number of times that clonotype was detected over "n" sequences analyzed) is shown. The length of the CDR3β region is according to Chothia et al., shown supported by two flanking framework regions. Bold indicates that the clonotype was simultaneously detected within the non-crossreactive BMLF1+ subset. Nucleotide sequences of all clonotypes analyzed can be found in Table 7.

D. Similar features between the cross-reactive and non-crossreactive TCR repertoires

i. Population analyses of TCR β-chain usage

Since the cross-reactive T cell population represents a subset of the M1- and BMLF1-specific T cell populations, we would expect that the cross-reactive TCR repertoire would share some features of both antigen-specific repertoires. Analyses of T cell lines derived from IM patient E1101 revealed that the Vβ repertoires of the cross-reactive T cell subsets included multiple Vβ families that were present in the non-crossreactive M1- and BMLF1-specific repertoires (Figure 24). The repertoire of the cross-reactive population which co-stained with M1- and BMLF1-loaded tetramers resembled that of the non-crossreactive M1-specific population because cells predominantly used Vβ17 (>80%). In contrast, the repertoire of the cross-reactive population which grew in response to M1 and bound only BMLF1-loaded tetramer used Vβ2 (12%), Vβ4 (18%), Vβ16 (6%), and Vβ22 (8%) but not Vβ17. We did not detect a dominant Vβ family within this cross-reactive population, suggesting that either the tetramer staining was somehow blocking the dominant Vβ-specific antibody staining or, more likely, that approximately 50% of this repertoire is comprised of other Vβ families which we do not have antibodies to detect. The same was true of the Vβ repertoire of the non-crossreactive BMLF1-specific population, which used Vβ2 (13%), Vβ4 (19%), and Vβ22 (10%) to account for less than 50% of the population. Overall, the M1-specific Vβ17 family was predominantly used by the sub-population of cross-reactive cells able to

efficiently bind the M1-loaded tetramer, while the BMLF1-specific Vβ families 2, 4 and 22 were predominantly used by the sub-population of cross-reactive cells better able to bind the BMLF1-loaded tetramer.

Similar analyses of other individuals included in our study also followed this trend. As was the case for IM patient E1101, the M1-specific T cell line derived from IM patient E1109 also contained two distinct, tetramer-defined, cross-reactive T cell subsets and the subset that co-stained with both tetramers predominantly used the Vβ17 family, which was also known to dominate the non-crossreactive M1-specific repertoire (Figure 25A, C). Once again, the Vβ17 family was absent from the repertoire of the cross-reactive subset that bound only BMLF1-loaded tetramer. Taken together, these data suggested that Vβ17 was required for stable binding of the M1-loaded tetramer, as has been previously reported (Lawson et al., 2001), and that cross-reactive TCRs with high avidity for M1 will likely use Vβ17. Based on our data, we can then infer that cross-reactive TCRs with lower avidity for M1, and therefore not staining with M1-loaded tetramer, may use non-Vβ17 families that are present in the BMLF1-specific repertoire. In general, previous studies would suggest that the BMLF1-specific TCR repertoires of most individuals include at least 1 of 4 common Vβ families (Vβ2, 4, 16, 22). Each of these 4 Vβ families was represented at least once among the cross-reactive repertoires of the 6 individuals investigated (Figure 25A, B).

Similarities between the cross-reactive and non-cross-reactive Vβ repertoires were also evident on a clonal level. We sub-cloned and sequenced the CDR3β regions of cross-reactive and non-crossreactive T cell subsets derived from healthy donor D-002.

This donor's non-crossreactive BMLF1-specific population predominantly used Vβ14 (66%), which was used by 57% of the cross-reactive population (Figure 25B, D). The most frequently detected Vβ14+ clonotype in the cross-reactive population (ID: B14.1, Jβ1.1, 7aa length) was the same clonotype that dominated the non-crossreactive BMLF1-specific repertoire of this donor (Figures 23 and 27A). On the other hand, this donor's non-crossreactive M1-specific population predominantly used Vβ17 (81%), which was used by <1% of the cross-reactive population (Figure 25B, C). This small population of Vβ17+ cross-reactive cells was surprisingly diverse at the clonal level, comprised of 42 unique clonotypes out of 55 total sequences analyzed (Figure 27B). There was no apparent skewing of the repertoire by one particular clonotype or any alteration of the Jβ hierarchy typical of resting M1+ Vβ17+ memory T cell populations (Naumov et al., 2005; Naumov et al., 2003). The cross-reactive Vβ17+ clonotypes of donor D-002 could be arranged in a power law-like distribution based on their frequency of detection, as has previously been used to describe the polyclonality of the Vβ17+ M1-specific memory T cell repertoire (Figure 27B, C) (Naumov et al., 2005; Naumov et al., 2003). In agreement with the literature, *RS* was the dominant CDR3β motif found within the non-crossreactive M1+ Vβ17+ repertoire (the predominant IRSS sequence was used by 25% of the unique clonotypes) and remained dominant with an even higher frequency within the cross-reactive population (the predominant IRSS sequence was used by 31% of the unique cross-reactive clonotypes), including the most frequently detected clonotype (ID: 17.3, frequency: 7% of total sequences) (Figure 27B, C) (Lehner et al., 1995; Moss et al.,

1991; Naumov et al., 1998). Interestingly, this cross-reactive Vβ17+ repertoire also had one clonotype in common with the non-crossreactive BMLF1-specific repertoire, which was the second most frequently detected Vβ17+ clonotype in the cross-reactive population (ID: B17.1, frequency: 5% of total sequences) and was the dominant Vβ17+ clonotype within the BMLF1-specific population (frequency: 84% of total sequences) having a CDR3β region that was 1aa residue longer (9aa length) than the IRSS-containing regions (Figures 23 and 27B).

ii. Population analyses of TCR α-chain usage

Vα repertoire analyses also revealed similarities between cross-reactive and non-crossreactive T cell subsets derived from healthy donor D-002. Non-crossreactive BMLF1-specific cells used Vα2, 10, 11, 15, 22 and 23, along with some other families that may have been at the limit of detection using this protocol (Figure 26B). All but Vα22 were also detected in the cross-reactive repertoire (Figure 26A). The Vα repertoire of non-crossreactive M1-specific cells also included a wide range of Vα families, most consistently including Vα1b, 2, 10 and 24, but with many additional Vα families being detected such as Vα6, 11, 15, 20 and 23 (Figure 26C). All of these families were also detected in the cross-reactive repertoire (Figure 26A). In order to compare the CDR3α lengths of cross-reactive and non-crossreactive T cells using the same Vα family, we ran the corresponding reactions on the same gel (Figure 26C). This further revealed that the cross-reactive cells often express similar CDR3α lengths to both non-crossreactive T cell populations. For example, the cross-reactive Vα10 spectratyping analysis closely

resembled that of the corresponding analysis on the non-crossreactive M1-specific population, while the cross-reactive Vα2 and Vα15 spectratyping analyses more closely resembled that of the corresponding analyses on the non-crossreactive BMLF1-specific population. As previously mentioned, Vα15 is a component of the public BMLF1-specific TCR and is likely important for interaction with the BMLF1 epitope. To verify the similarity of these repertoires on a clonal level, we sub-cloned and sequenced the Vα15+ TCRs from all three T cell subsets (Figure 28). We detected the identical dominant Vα15+ clonotype (ID: B1) in both the cross-reactive (frequency: 70% of total sequences) and non-crossreactive BMLF1-specific populations (frequency: 76% of total sequences) (Figure 28A, B).

iii. TCR structure of cross-reactive T cell clones

In light of so many similarities between the cross-reactive and non-crossreactive repertoires, we sought to co-sequence the TCR α- and β-chains of cross-reactive T cell clones in order to determine if it was the pairing of a BMLF1-specific α-chain with an M1-specific β-chain, or vice versa, that enabled a cross-reactive TCR to engage both epitopes. In preliminary experiments, we sequenced three cross-reactive T cell clones that co-stained with both tetramers (Table 8). Two of the clones used a TCR with α- and β-chains that were both detected in the same epitope-specific repertoire. Clone 1D4 used Vα1b/Jα42 (AV-NSGGGSQGNL-IF) and Vβ17/Jβ2.7 (AS-SSRSSYEQ-YF), and both CDR3 sequences were previously detected within M1-specific T cell populations, but in experiments that did not exclude potentially cross-reactive cells (Naumov et al., 2005) (Naumov et al., manuscript in preparation). Interestingly, the CDR3 regions of both

Table 8. TCR structure of cross-reactive T cell clones.

ID	Vβ family	Jβ family	CDR3β sequence	Vα family	Jα family	CDR3α sequence
1D4	17	2.7	AS-S**SRS**SYEQ-YF	1b	42	AV-N**SG**GGSQGNL-IF
4E5	16	2.5	AS-SQ**SPGG**TQ-YF	15	3	AE-YSSASKI-IF
1F8	2	1.3	SA-R**SGV**GNTI-YF	15	11	AE-**DRD**STL-TF

T cell clones were each derived from a single T cell that co-stained with M1- and BMLF1-loaded tetramers and that was cultured for 2 weeks. The V region nomenclature is according to (Arden et al., 1995) and the CDR3 junction region is shown flanked by two framework regions. Bolded residues are the N-nucleotides added to the junction region and, in the case of the β-chain, include the D region.

chains were extended by non-bulky aa residues, glycine and serine, which may have enhanced the flexibility of this M1-specific TCR and allowed it to engage the BMLF1 epitope (Abergel and Claverie, 1991; Bentley and Mariuzza, 1996). Clone 4E5 used Vα15/Jα3 (AE-YSSASKI-IF) and Vβ16/Jβ2.5 (AS-SQSPGGTQ-YF), and both CDR3 sequences were previously detected within BMLF1-specific T cell populations (Figures 23 and 28). Once again, serine and glycine residues were prominent in both chains. Finally, clone 1F8 used Vα15/Jα11 (AE-DRDSTL-TF) and Vβ2/Jβ1.3 (SA-RSGVGNTI-YF), and neither of these CDR3 sequences appears to be a common component of M1- or BMLF1-specific TCRs. This CDR3α region does not fit the predicted BMLF1-specific Vα15+ public motif (Table 2) and, in our study, was only detected in the cross-reactive TCR repertoire (Figure 28). While the CDR3β region does resemble a predicted BMLF1-specific Vβ2+ public motif (Table 2), the non-germline encoded serine residue is a rare component. It is possible that T cell clones expressing this serine residue in their CDR3β region are better apt to interact with the M1 epitope due to its juxtaposition to the arginine residue, the two most conserved residues of the *RS* public motif for contacting the M1 peptide according to the crystal structure of *JM22* TCR/M1/HLA-A2 (Stewart-Jones et al., 2003).

E. The cross-reactive TCR repertoire is unique to each individual

Cross-reactive TCR Vβ analyses of 6 different individuals revealed the wide range of Vβ families capable of recognizing both M1 and BMLF1 epitopes, but these analyses also demonstrated the individual variability in the organizational pattern of each cross-reactive TCR repertoire (Figure 25). It appeared that the specific combination of

BMLF1-specific Vβ families used by each individual influenced the specific combination of cross-reactive Vβ families used by that individual. For example, the non-crossreactive BMLF1-specific repertoire of donor D-042 included Vβ4 and Vβ16, but not Vβ2 or Vβ22, and the cross-reactive repertoire of this donor reflected that by including one of the two Vβ families known to be present, Vβ4 (Figure 25B, D). Thus, the composition of the non-crossreactive Vβ repertoire clearly influenced the organization of the cross-reactive Vβ repertoire. The individual variability associated with both BMLF1-specific and, through association, cross-reactive Vβ repertoires stems from the private specificity of each individual's pool of precursor T cells (naïve and memory) capable of activation by the BMLF1 epitope. Thus, there did not appear to be a public V region component of cross-reactive TCRs, and the individual variability of each cross-reactive repertoire may contribute to the individual variability in disease severity associated with cross-reactive T cell responses.

F. Chapter V summary

In this study, we aimed to characterize the structure(s) of this specific cross-reactive TCR and the organization of the cross-reactive TCR repertoire. The epitope sequence of M1 (GILGFVFTL) shares only 3 of 9 aa residues with that of BMLF1 (GLCTLVAML), at least 1 of which is important for MHC binding (Engelhard, 1994). Hence, this is not an obvious case of molecular mimicry, and it is likely that the TCR elements used to engage M1 differ from those used to engage BMLF1. TCR analyses of cross-reactive T cell populations revealed that a wide range of Vα and Vβ families can mediate interaction with both epitopes. In addition, comparisons of the cross-reactive

TCR repertoires of several individuals revealed that each repertoire remained unique to the individual, relying heavily on the clones present in that individual's private M1- and BMLF1-specific repertoires. However, TCRs with longer CDR3 regions, often extended by non-bulky aa residues such as glycine and serine, were more prominent within the cross-reactive repertoire. These unique features presumably make them more permissible to interactions with these two dissimilar epitopes. Thus, the cross-reactive repertoire was more diverse than either non-crossreactive repertoire alone because it included both high and low frequency clones present in the non-crossreactive M1-specific and non-crossreactive BMLF1-specific repertoires. If the total number of CDR3 sequences analyzed within Vβ families 14 and 17 are combined, and we compare the total number of unique Vβ-defined clonotypes found in each of the three repertoires of healthy donor D-002, the cross-reactive repertoire (47 unique clonotypes out of 78 total sequences) was clearly more diverse than that of the M1-specific (41 unique clonotypes out of 146 total sequences) or BMLF1-specific (13 unique clonotypes out of 115 total sequences) repertoires. These data suggest that there is great potential for activating M1-specific memory cells during an acute EBV infection, and the private specificity of each cross-reactive TCR repertoire provides at least one explanation for the individual variability we see in the detection of cross-reactive T cell responses with specificity for M1 and BMLF1.

CHAPTER 6:

DISCUSSION

Based on work using murine models of heterologous viral infection, we hypothesized that cross-reactive CD8 T cell responses are common components of the human immune response to viral infection and can influence the outcome of an infection. Within our model system, we hypothesized that Epstein-Barr virus encodes multiple T cell epitopes that can activate many cross-reactive T cell responses, especially within individuals with diverse memory T cell pools due to a long history of previous virus exposures. We predicted that, collectively, these cross-reactive T cell responses promote the development of infectious mononucleosis, an immune-mediated pathology associated with acute EBV infection. This thesis research supports our hypothesis, as we identified five putative cross-reactive T cell responses that could occur during an acute EBV infection, including three that would involve the activation of cross-reactive influenza virus-specific memory T cells. Furthermore, we showed that, at presentation with IM, patients frequently had an increase in the number of (IV)M1-specific T cells in their blood, suggesting that a subset of M1-specific memory cells proliferated in response to this acute EBV infection. We defined at least one cross-reactive T cell response in two IM patients, with specificity for (IV)M1 and (EBV)BMLF1. Our observation that the M1-specific TCR repertoires of these two patients were highly skewed was an indication of cross-reactive T cell expansions. This cross-reactive T cell response may have influenced the severity of IM, as one of the patients presented as a particularly severe case, and we have demonstrated that these cross-reactive T cells can produce multiple

pro-inflammatory cytokines upon stimulation with either epitope. While a direct correlation between the presence of cross-reactive T cell responses and IM severity will require further investigation into the contributions made by cross-reactive responses with alternative specificites, our data implies that cross-reactive T cells contribute to the lymphoproliferation characteristic of IM. The data presented here affirmed many of the observations made on CD8 T cell cross-reactivity and heterologous immunity using a controlled murine model system and revealed a significant role for cross-reactive T cell responses on a human disease state.

A. A matrix of cross-reactive T cell specificities

Upon screening for cross-reactive T cell responses involving EBV-derived HLA-A2-presented epitopes, we detected 5 different cross-reactive specificities and 4 of the 5 specificities involved the EBV-BMLF1$_{280\text{-}288}$ epitope (Chapter III, Figure 29). It is still unclear what elements of the BMLF1 epitope structure are used for cross-reactive TCR interaction, but it is possible that it varies since the majority of the epitopes co-recognized, derived from EBV and IV, shared little to no sequence homology with BMLF1. Such a complex matrix of cross-reactive specificity patterns has also been observed in the murine system, where 5 different cross-reactive specificities involved the VV-A11R$_{198\text{-}205}$ epitope (Figure 30) (Cornberg et al., 2005a). The co-recognized epitopes were derived from VV, PV and LCMV and the majority of them shared no more than 3 of the 8 aa residues in common with the A11R epitope sequence. Interestingly, the specific cross-reactive pattern that emerged appeared to depend on the sequence of viral infections, where A11R-specific T cell lines derived from LCMV-immune mice revealed

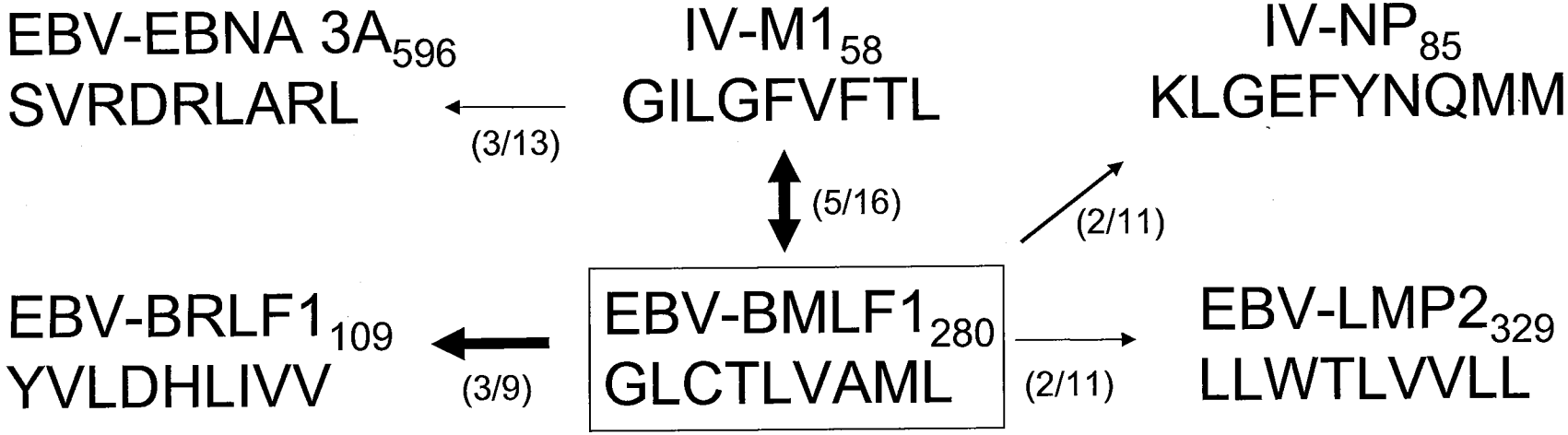


Figure 29. Matrix of T cell cross-reactivity within a human system of viral infection. Based on intracellular IFNγ production, CD8 T cell lines specific for one epitope responded to alternative epitopes derived from both EBV and IV. All epitopes are presented by HLA-A2. The thickness of the arrow reflects number of individuals within our study showing evidence of each cross-reactive specificity pattern, while the absence of an arrow indicates there was no evidence of a cross-reactive response involving those epitopes in our study. This figure was derived from data presented in Chapter III of this thesis.

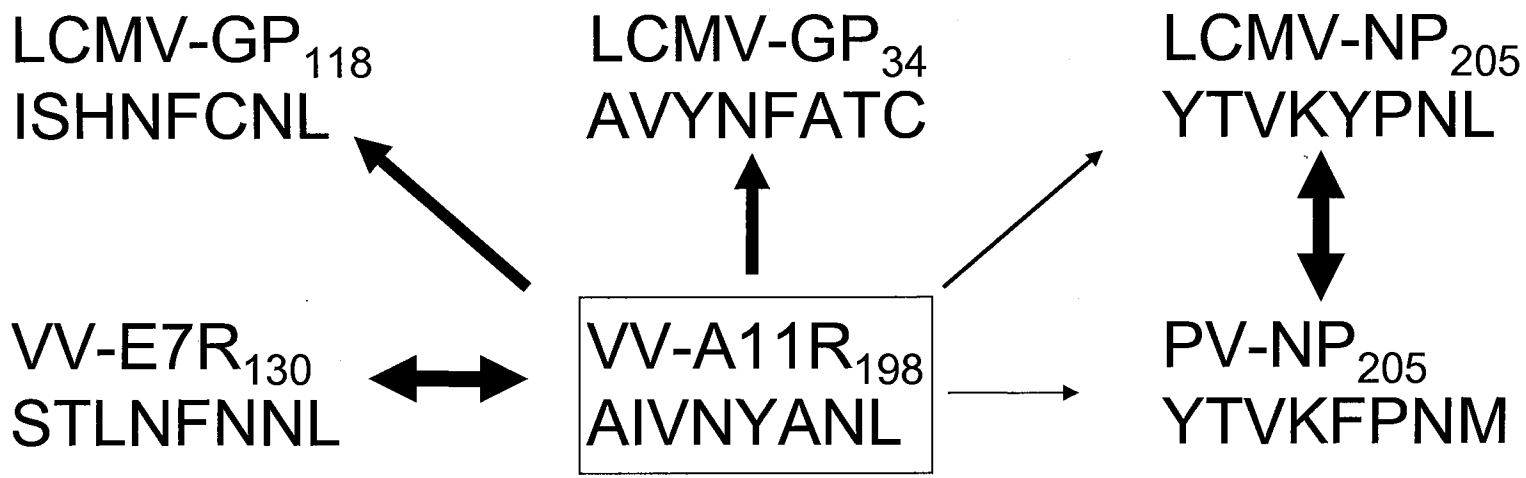


Figure 30. Matrix of T cell cross-reactivity within a murine system of viral infection. Based on intracellular IFNγ production, CD8 T cells specific for an epitope derived from VV (A11R) can recognize and respond to epitopes derived from other VV-derived proteins as well as epitopes derived from other unrelated viruses, LCMV and PV. All epitopes are presented by H2-K^b. The thickness of the arrow reflects the magnitude of the cross-reactive T cell response. This figure was derived from unpublished data (Cornberg et al., 2005a).

more frequent cross-reactive recognition of LCMV-GP epitopes as opposed to those lines derived from VV-immune mice that more frequently demonstrated cross-reactivity with VV-E7R. Both of these studies, one in mice and one in humans, reveal how prevalent CD8 T cell cross-reactivity can be and emphasize the fact that having epitope sequence similarity, referred to as molecular mimicry, is not the only mechanism of T cell cross-reactivity. A TCR can undergo large conformational changes to accommodate ligands that one might not predict it could bind (Garcia et al., 1998). This point has been clearly demonstrated by Boen and colleagues using a high throughput technology for screening over 100,000 candidate peptide ligands for a single TCR, where the three stimulatory peptides identified could not have been predicted based on sequence similarity to the native ligand (Boen et al., 2000).

B. The private specificity of cross-reactive T cell responses

One of the most consistent observations made regarding heterologous immunity was the variable pattern of T cell cross-reactivity that emerged. For instance, LCMV-immune mice were protected from VV challenge, and evidence suggested that cross-reactive memory CD8 T cell responses played a role in this protection (Selin et al., 1998). However, only 50% of LCMV-immune mice challenged with VV mounted a cross-reactive T cell response specific for the LCMV-$NP_{205-212}$ epitope, while the other mice had no evidence of a cross-reactive response or mounted a cross-reactive response with an alternative specificity (Figure 31) (Kim et al., 2005). Similar observations were made in the human system. A cohort of 8 IM patients all had M1-specific T cells in their peripheral blood, taken as evidence of exposure to IV prior to EBV infection. However,

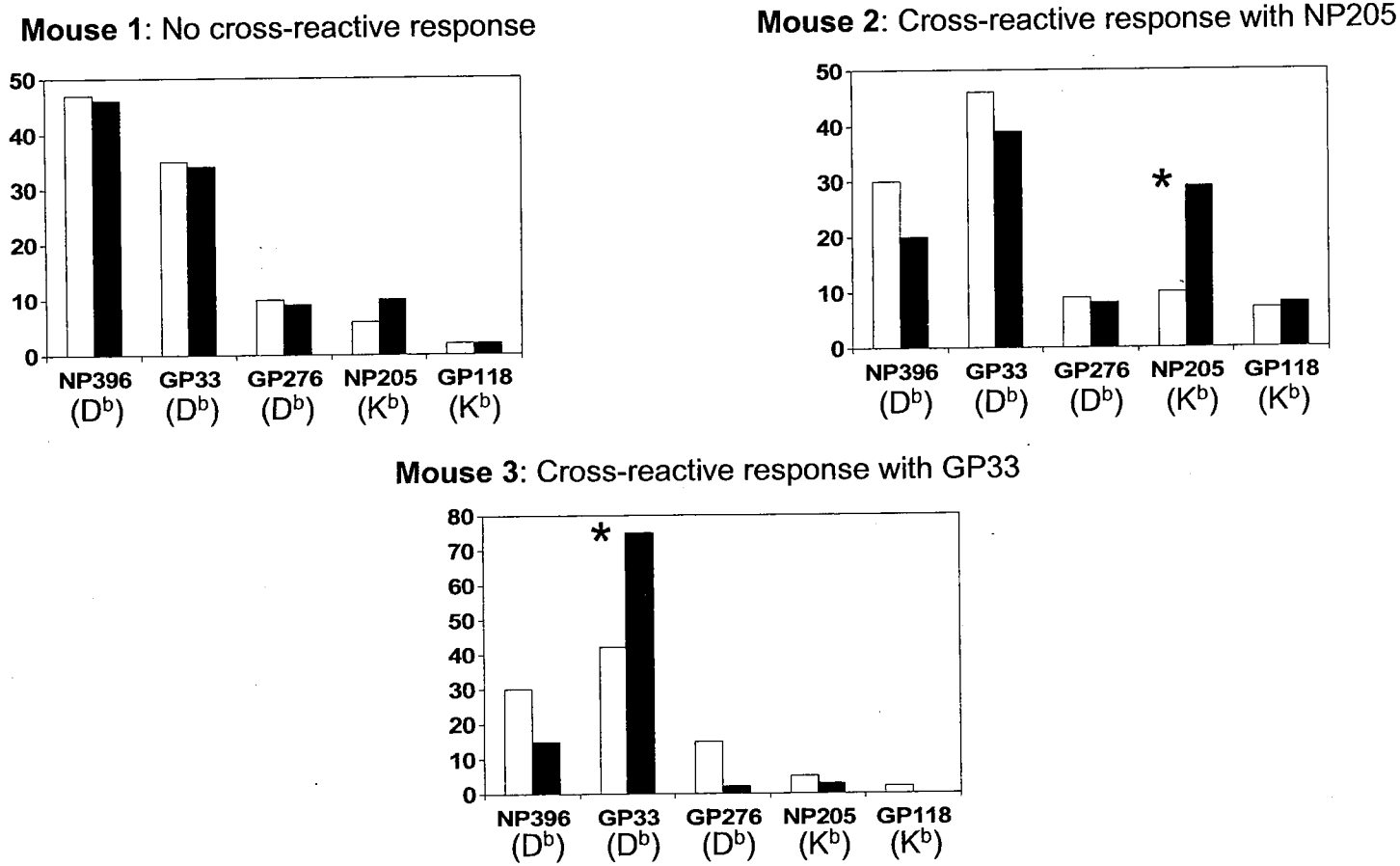


Figure 31. Private specificity determines the pattern of cross-reactive CD8 T cell responses of LCMV-immune mice challenged with VV. This data was presented in Kim et al. 2005 J Exp Med 201:523-533, and illustrates the relative proportion (%) of LCMV-specific CD8 T cells producing IFNγ in response to stimulation with one of five different LCMV-derived peptides before (white bars) and after (black bars) a VV challenge. The VV infection skewed the epitope-specific hierarchy of each LCMV-immune mouse differently depending on the cross-reactive response that emerged (*).

only 5 patients had evidence of M1-specific T cell expansion as a result of EBV infection and only 2 patients within that group showed evidence of a cross-reactive T cell response with specificity for M1 and BMLF1 (Chapter IV). These data suggested that not all IV-immune individuals have cross-reactive M1-specific T cells that co-recognize EBV-derived antigens and that individuals that do mount a cross-reactive response still differ by the clonotypic composition of their cross-reactive T cell populations.

The private specificity of a host's antigen-specific TCR repertoire can influence the magnitude and specificity of the cross-reactive T cell response. Work from our lab revealed that the (LCMV)NP-specific T cell response of each mouse was comprised of unique proportions of NP-specific Vβ families (Vβ16, 5 and 8.2) and unique clonotypes within those Vβ families, which may explain the observed variability in the specificity of each cross-reactive response (Kim et al., 2005). Indeed, using adoptive transfer experiments, we demonstrated that LCMV-immune cells derived from one donor mouse dictated the cross-reactive response of three recipient mice challenged with VV, justifying the argument that the private epitope-specific repertoire of the immune-host determined the magnitude and specificity of the cross-reactive response. Although such controlled experiments are not possible in the human system, we know that the M1-specific memory TCR repertoire of each individual is comprised of hundreds of unique clones (Naumov et al., 2005; Naumov et al., 2003). In addition, analyses of the cross-reactive TCR repertoires of 6 different individuals revealed that each included a unique pattern of Vβ usage that was largely influenced by the unique pattern of that individual's non-crossreactive M1 and BMLF1-specific TCR repertoires (Chapter V). Thus, cross-

reactive T cell clones are a subset of a clonotypically diverse, private, epitope-specific TCR repertoire. If the cross-reactive clones of a given specificity represent only a small proportion of that epitope-specific repertoire, then the cross-reactive response is likely to be of a lower magnitude than that which occurs when the cross-reactive clones of a given specificity represent a large proportion of that epitope-specific repertoire.

C. A cross-reactive T cell population is comprised of clones with different functional avidities and different TCR structures

CD8 T cells can become cytotoxic, secrete cytokines, and proliferate following their activation. Extensive research on T cell activation using altered peptide ligands has resulted in the knowledge that T cell effector functions can be segregated and are likely independently regulated by mechanisms that are still unclear, but the quantity and quality of the TCR-mediated signal appear to play a role. Through the study of natural, virus-induced, cross-reactive CD8 T cell responses, our lab has demonstrated that a cross-reactive stimulation is more efficient at eliciting cytokine production than proliferation. For instance, a subset of at least three different LCMV epitope-specific memory T cell populations produced IFNγ 3 days following exposure to VV, suggesting the activation of a wide range of cross-reactive responses; however, by 12 days after a VV challenge, there was a significant shift in the typical LCMV epitope-specificity hierarchy, an indication of the selective proliferation of cross-reactive T cells having only certain epitope-specificities (Chen et al., 2001). A similar trend was observed using a second model system of heterologous immunity, that involving LCMV and PV, whereby a relatively equal proportion of PV-specific T cells produced IFNγ in response to stimulation with

either the (PV)NP_{205} or (LCMV)NP_{205} peptides (Figure 32) (Cornberg et al., 2005b). While this result suggested most (PV)NP-specific cells could function in response to (LCMV)NP stimulation, longitudinal experiments that followed the initially diverse NP-specific TCR repertoire of a PV-immune mouse after a challenge with LCMV revealed that the NP-specific repertoire narrowed, dominated by T cells expressing only certain Vβ families (Figure 32) (Cornberg et al., 2005b).

In the human studies presented here, we also saw a hierarchy of functions in response to a cross-reactive stimulation. We demonstrated that a greater proportion of T cells responded to cross-reactive stimulation by producing MIP-1β than by producing IFNγ, and an even smaller proportion produced TNFα (MIP-1β>IFNγ>TNFα) (Chapter IV). Although it was not tested directly, we also found that cross-reactive stimulation was not efficient at inducing T cell proliferation in vitro, as evidenced by the often low frequencies of cross-reactive cells in bulk cultures and the lack of long-term cross-reactive T cell clones.

We observed differences in the functional response to M1 versus BMLF1 peptide stimulation using two distinct cross-reactive T cell populations (Chapter III). The cross-reactive population within an M1-specific T cell line produced some IFNγ and high levels of MIP-1β to BMLF1 peptide stimulation. In contrast, the cross-reactive population within a BMLF1-specific T cell line produced only minimal levels of IFNγ and no MIP-1β to M1 peptide stimulation. The functional differences between these two cross-reactive T cell populations may stem from a difference in the avidity of the interaction between the TCR and M1 versus the TCR and BMLF1, which can influence the quantity

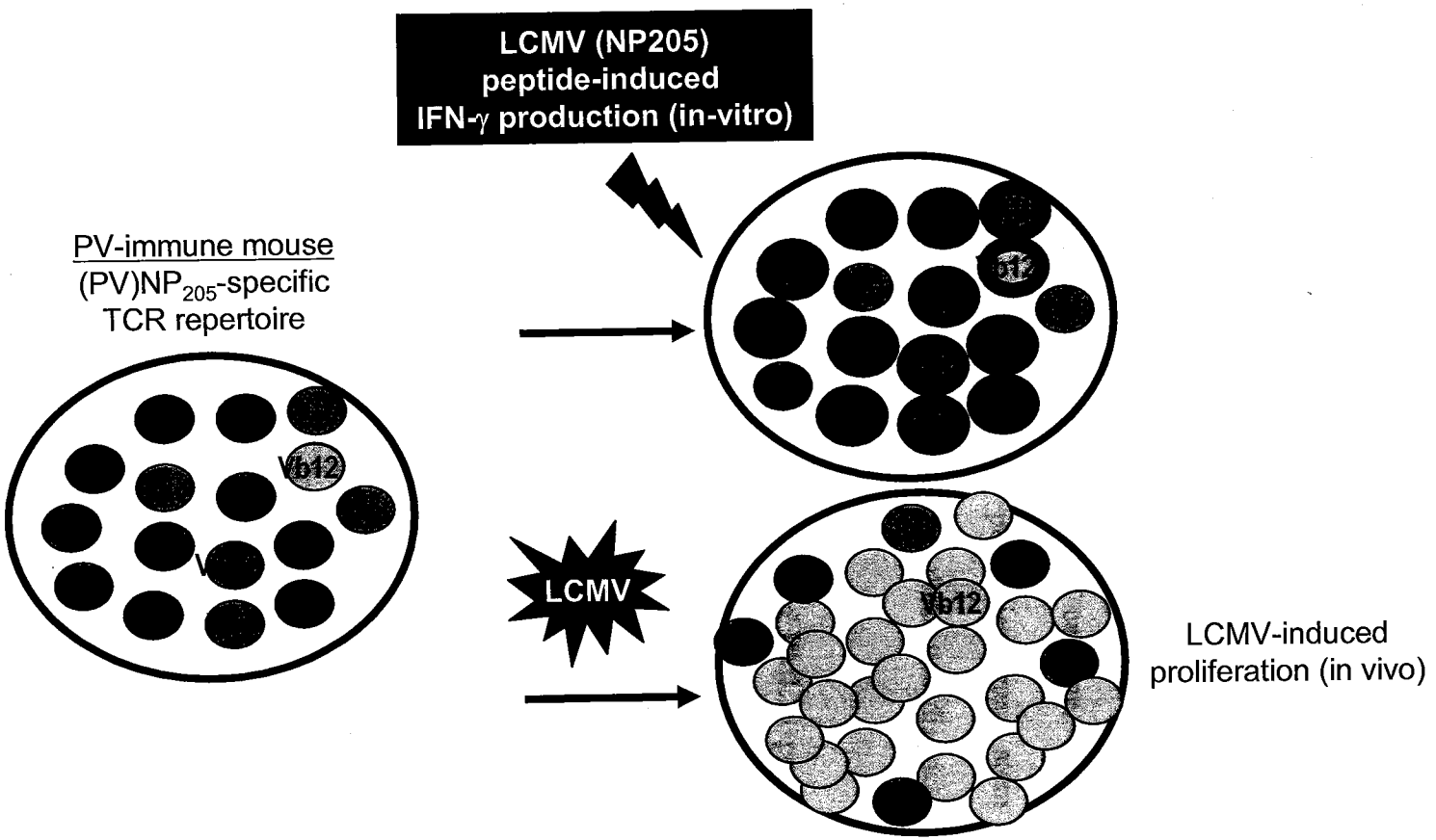


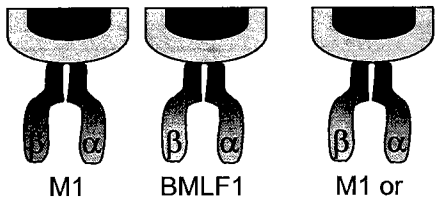
Figure 32. Cross-reactive T cell responses are more restricted at proliferation than at cytokine production. This model is based on unpublished data (Cornberg et al., 2005b) and illustrates that, in this murine system, an intracellular IFNγ assay detected a wider range of cross-reactive T cell responses in vitro than was detected after a heterologous viral infection induced clonal T cell expansions in vivo.

and quality of the TCR signal. We did find a difference between these two populations in the relative avidity for their respective alternative ligand. Based on IFNγ production, the cross-reactive population within the BMLF1-specific line had a higher avidity for its alternative ligand (M1) than did the cross-reactive population within the M1-specific line for its alternative ligand (BMLF1) (Chapter III). While these avidity differences may explain our results, they would go against the paradigm that a higher avidity interaction results in greater functional capability, unless the M1 peptide is acting as a partial antagonist in the context of a BMLF1-specific T cell line. Alternatively, the functional differences between these two T cell populations may stem from the qualitative difference in the TCR signal generated from these two distinct ligands, irrespective of avidity.

Within a T cell line cultured with M1 and BMLF1 peptides simultaneously, we were able to compare the functional response of two distinct cross-reactive subsets to the same peptide ligand (Chapter IV). The T cell subset that co-stained with both M1- and BMLF1-loaded tetramers produced a hierarchy of cytokines (MIP-1β>IFNγ>TNFα) in response to M1 peptide stimulation. In response to this same M1 peptide stimulation, the T cell subset that only stained with the BMLF1-loaded tetramer produced a lower level of cytokines overall but exhibited the same profile, in that more MIP-1β was produced than IFNγ, and no TNFα production was detected. Based on IFNγ production, the relative avidity for the M1 peptide was higher in the double-tetramer positive subset than in the single-tetramer subset (Chapter IV). Since, in this case, the stimulating ligand remained

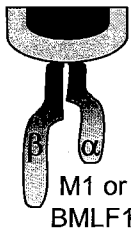
the same, the differences in the avidity and in the functional response between these two distinct T cell populations may stem from differences in their TCR repertoires.

Our analyses of cross-reactive TCR repertoires would indeed suggest that multiple different TCR structures can co-recognize M1 and BMLF1, as they included a wide range of Vα and Vβ families (Chapter V). Such a diverse array of cross-reactive TCR structures could indicate that many different structural mechanisms co-exist to mediate this cross-reactive interaction and that each does so with its own unique avidity. As mentioned in the introduction to this thesis, there are at least three potential models of a cross-reactive interaction (Figure 1). The fact that the bulk of our data on cross-reactive TCR structure was generated using T cell populations, rather than T cell clones, precludes us from determining if a cross-reactive T cell expresses two different TCRs and uses them to interact with both M1 and BMLF1. There is little reason to suspect that molecular mimicry explains this particular cross-reactive interaction; the two epitopes do share a valine residue at position 6 of their sequence that appears to be important for contact with M1- and BMLF1-specific TCRs (Annels et al., 2000; Stewart-Jones et al., 2003), but it is unlikely that this shared residue is solely responsible for the specificity of the interaction. It is more likely that different elements of the cross-reactive TCR are used to interact with M1 versus BMLF1, a mechanism we refer to as alternative recognition. Our cross-reactive TCR repertoire analyses support three different models of alternative recognition (Figure 33). For instance, a cross-reactive TCR may be comprised of an M1-specific β-chain and a BMLF1-specific α-chain, or vice versa, where it predominantly uses the β-chain to mediate interaction with M1 and the α-chain



MODEL 1: A CROSS-REACTIVE TCR EXPRESSES A UNIQUE COMBINATION OF α- and β-CHAINS.

eg.) The public specificity of an M1-specific TCR repertoire includes Vβ17 and the public specificity of a BMLF1-specific TCR repertoire includes Vα15. We have evidence that the cross-reactive TCR repertoire includes both Vβ17 and Vα15, but cross-reactive T cell clones are necessary for determining whether these two V-specific families can pair.



MODEL 2: A CROSS-REACTIVE TCR EXPRESSES A UNIQUE CDR3 SEQUENCE, PERHAPS OF GREATER LENGTH AND COMPRISED OF NON-BULKY RESIDUES, THAT PROVIDES ADDED FLEXIBILITY IN ORDER TO MAKE A UNIQUE AND STABLE INTERACTION WITH EITHER M1 OR BMLF1.

eg.) Cross-reactive Vβ14+ TCR, CDR3β: AS-SSGSSGYYNEQ-FF
(typical BMLF1-specific CDR3β: AS-GRTNTEA-FF)
eg.) Cross-reactive Vβ17+ TCR, CDR3β: AS-SIETTSGSSYNEQ-FF
(typical M1-specific CDR3β: AS-SIRSSYEQ-YF)

MODEL 3: A SUBSET OF TCRS WITHIN THE ANTIGEN-SPECIFIC REPERTOIRE HAPPENS TO EXPRESS A CDR3 SEQUENCE WITH FEATURES OF AN M1- AND A BMLF1-SPECIFIC MOTIF.

eg.) BMLF1-specific Vβ2+ repertoire

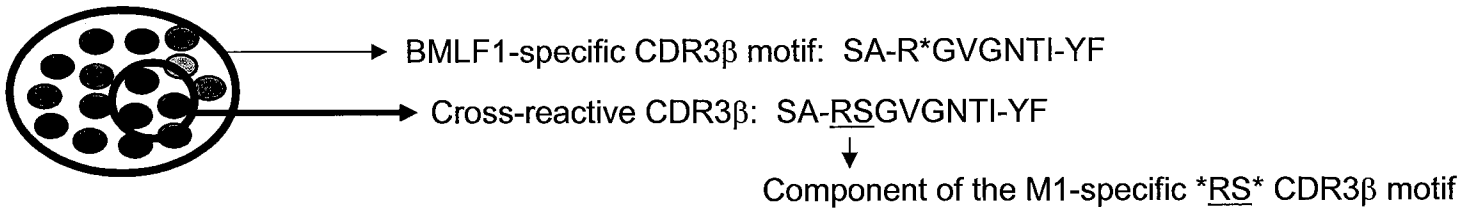


FIGURE 33. Models of cross-reactive TCR-mediated alternative recognition of M1 and BMLF1. Models are based on data presented in Chapter V of this thesis.

to interact with BMLF1. This model is plausible knowing that the public specificity of an M1-specific repertoire includes Vβ17 and that the β-chain mediates much of the contact with the M1 peptide, whereas the public specificity of a BMLF1-specific repertoire includes Vα15, which is suspected to mediate contact with BMLF1 due to its ability to pair with multiple Vβ families. A second model would include cross-reactive cells that express unique TCRs, not typically detected within M1- or BMLF1-specific populations. We showed several examples of cross-reactive TCRs that expressed longer CDR3 loops and that were often comprised of non-bulky aa residues, features which may confer added flexibility to the TCR and allow it to interact with two structurally dissimilar ligands. We also detected cross-reactive TCRs that fit a third model of alternative recognition, those which expressed a CDR3 sequence having features of both M1- and BMLF1-specific motifs. In addition to the expression of Vβ17, the public specificity of an M1-specific repertoire includes a CDR3β motif of *RS*, the arginine and serine being particularly important for contact with M1. Interestingly, one of the cross-reactive clones we isolated expressed a BMLF1-specific Vβ family, Vβ2, but the CDR3β sequence was somewhat atypical in that it included a non-germline encoded serine residue that was positioned after a conserved arginine residue. Therefore, this cross-reactive TCR CDR3β loop included an 'RS' sequence that could potentially mediate contact with M1, while the rest of the sequence fit the motif of other known BMLF1-specific TCRs. Overall, our data suggest that multiple different cross-reactive TCR structures exist and support at least 3 different models for the interaction between a cross-reactive TCR and these two dissimilar ligands, M1 and BMLF1.

D. The influence of cross-reactive T cell responses on disease pathogenesis and the memory TCR repertoire

From experiments performed in a murine model system, we know that cross-reactive LCMV-specific memory T cell responses can alter the disease pathology associated with acute VV infection. In contrast to the characteristic edema in the lungs of naïve mice challenged intranasally with VV, the lungs of LCMV-immune mice were infiltrated with LCMV-specific memory T cells and in some cases developed bronchiolitis obliterans, a pathology where the bronchioles were obstructed with fibrin and inflammatory cells (Chen et al., 2001). If delivered intraperitoneally, VV caused immunopathological lesions in the visceral fat of LCMV-immune mice, which were areas of necrosis that were also infiltrated with LCMV-specific memory and inflammatory cells (Selin et al., 1998; Yang et al., 1985). It was suspected that the high levels of IFNγ produced by these immune cells promoted both pathologies and the role of TNFα is currently being investigated (Chen et al., 2001; Selin et al., 1998).

In this preliminary study of patients with acute EBV infection, we reported one case where we detected a high level of cross-reactive T cells in the peripheral blood that co-stained with M1- and BMLF1-loaded tetramers (Chapter IV). This patient presented with severe clinical signs and symptoms of infectious mononucleosis, which may be caused by the overall increased level of cytolysis and cytokines at the site of infection due to the contribution of these and other activated cross-reactive cells. In vitro, we have demonstrated that cross-reactive cells that co-stain with both M1- and BMLF1-loaded tetramers can lyse target cells presenting either epitope as well as produce MIP-1β, IFNγ,

and TNFα in response to either epitope (Chapter IV). Interestingly, all three cytokine levels are elevated in the serum and tonsils of IM patients compared to healthy controls (Foss et al., 1994; Iwatsuki et al., 2004; Nakayama et al., 2004). MIP-1β can be readily secreted because it is preformed and stored within human CD8 T cells and, therefore, even a low avidity cross-reactive interaction is likely to stimulate its release (Zaitseva et al., 2001). MIP-1β acts to broaden the immune response by recruiting other immune cells to the site of infection, and an increase in the number of activated immune cells will likely exacerbate the inflammatory response and increase the severity of IM (De Rosa et al., 2004). We showed that fewer cross-reactive T cells secreted high levels of TNFα, which suggested that a higher avidity interaction was required to initiate its release from the cell. However, an overall increase in the number of responding T cells capable of secreting TNFα at the site of infection could be harmful to the host and promote the clinical symptoms of IM (Vassalli, 1992; Xu et al., 2004). Although many more patients will need to be investigated, these data follow the trend observed in the murine system, which is that cross-reactive T cells have the potential to mediate virus-associated disease pathology. However, if many cross-reactive T cell responses of different specificities collectively promote this immune-mediated disease, as we would predict, then a true correlation may depend on our ability to define and quantify other cross-reactive specificities in these IM patients.

The activation of cross-reactive memory cells also has an impact on the memory TCR repertoire and, consequently, the immune response to subsequent infections. For instance, a cross-reactive T cell population was previously defined that recognized two

distinct epitopes derived from the nucleoproteins of LCMV and PV (Brehm et al., 2002). The normally sub-dominant response to PV-$NP_{205-212}$ became dominant upon acute PV infection of LCMV-immune mice. Following heterologous infection, TCR analyses of this cross-reactive T cell population revealed the development of a more restricted NP-specific TCR repertoire compared to that prior to the infection (Cornberg et al., 2005b). This cross-reactive repertoire was most often comprised of fewer Vβ families and fewer clonotypes within those Vβ families, suggesting that very few cross-reactive clones proliferated in vivo following heterologous virus infection. While we were unable to compare the M1-specific TCR repertoire of the same individual before and during their acute immune response to EBV infection, we did find that the two IM patients with detectable cross-reactive T cells specific for M1 and BMLF1 in their peripheral blood had M1-specific TCR repertoires that were largely skewed from the conserved clonotypic pattern typical of an M1-specific TCR repertoire in a resting state (Chapter IV) (Naumov et al., 2005). Vβ17+ clonotypes using Jβ2.3 preferentially expanded in patient E1101, while those using Jβ2.1 preferentially expanded in patient E1178. While the clonal difference between these two patients was reflective of the private specificity of their repertoires, both cases appeared to result in a less diverse M1-specific repertoire than the typically polyclonal M1-specific repertoire of healthy IV-immune donors, particularly in the case of E1178 where as few as two unique clonotypes were detected. Based on the usual dominance of Vβ17/Jβ2.7 clones, the normally sub-dominant Vβ17/Jβ2.3 and Vβ17/Jβ2.1 clones may have a relatively lower avidity for the M1 epitope. Thus, skewing of the normal hierarchy of M1-specific clones by cross-reactive clonal T cell

expansions responding to acute EBV infection may leave these two individuals more vulnerable to a re-infection with IV and subject to alterations in the disease pathology associated with re-infection.

E. Cross-reactive T cells commonly contribute to human immune responses

There is likely a role for cross-reactive T cells in the immune response to many human viral infections. The research presented in this thesis alone demonstrated a cross-reactive T cell response with specificity for two immunodominant epitopes derived from two of the most common human viruses among people that share one of the most common MHC Class I alleles in the U. S.. T cells with this cross-reactive specificity could be detected in 38% of healthy donors with previous exposure to both viruses, suggesting that these cells can be maintained in memory. In 25% of the patients investigated, T cells with this particular cross-reactive specificity contributed to the immune response to acute EBV infection and likely promoted the development of the immune-mediated infectious mononucleosis syndrome (Chapter IV). Furthermore, T cells with this cross-reactive specificity can express a variety of TCR structures, including at least 10 different Vβ families and at least 9 different Vα families (Chapter V). Thus, the potential is high for activating a cross-reactive T cell clone having this specificity during infection with either virus.

As is true with EBV, acute HCV infection is often asymptomatic, but when clinical symptoms manifest, they appear to be immune-mediated. HCV encodes an epitope, $NS3_{1073\text{-}1081}$, that can activate influenza A virus $NA_{231\text{-}239}$-specific memory cells (Wedemeyer et al., 2001). However, unlike the cross-reactivity between EBV-BMLF1

and IV-M1, which are only 33% similar in sequence, HCV-NS3 and IV-NA are 78% similar in sequence. The activation of these cross-reactive NA-specific memory cells enhanced the magnitude, but restricted the specificity, of the CD8 T cell response to HCV and resulted in severe liver pathology (Urbani et al., 2005). Despite their strength in numbers, these cross-reactive cells were unable to sufficiently clear the virus and the patients developed persistent HCV infection. Hence, there are now at least two examples of cross-reactive T cell responses promoting disease pathology associated with two different viral infections.

Dengue virus infection provides yet another example of a direct correlation between disease severity and the magnitude of the T cell response (Mongkolsapaya et al., 2003; Zivna et al., 2002). Activated T cells are thought to promote the development of Dengue hemorrhagic fever through the production of harmful inflammatory cytokines, such as TNFα, known to cause plasma leakage (Rothman and Ennis, 1999). This disease is more commonly associated with secondary Dengue virus infections with a different serotype to which the individual has no neutralizing antibodies, but does have evidence of cross-reactive CD8 T cells that can co-recognize epitopes derived from both the primary and secondary Dengue virus serotype (Mongkolsapaya et al., 2003).

Thus, CD8 T cell cross-reactivity may be the rule rather than the exception during a human immune response. No one is naïve, and memory T cells specific to previously encountered pathogens reside in the body awaiting any opportunity for re-activation to ensure their long-term survival in a dynamic immune system. The contribution made by cross-reactive T cells to the immune response can easily go unnoticed, especially when

the result is beneficial. As memory cells, they are the first at the site of infection, are at higher precursor frequencies, and have less requirements for their full activation than do naïve T cells. If these cross-reactive T cells are efficient at clearing the pathogen, then often times an infection may be subclinical and will resolve quickly. It is when these cross-reactive T cells are inefficient at clearing virus that they cause damage to the host. They can prolong an infection, perhaps indefinitely in the face of a persistent virus, and can inhibit an otherwise efficient primary immune response from occurring. They may continue to fight a losing battle, secreting multiple cytokines that only exacerbate a tissue-damaging inflammatory response directly or indirectly through the recruitment of even more immune cells to the site of infection. In cases such as these, the contribution of a cross-reactive T cell response may become more obvious, resulting in distinct disease pathologies.

Due to different histories of infection and the private specificities of T cell repertoires, establishing a direct cause and effect relationship between a given cross-reactive T cell response and disease state may be very difficult with human studies. That is why murine models are so important to the research of cross-reactive T cell responses. Thus far, the concepts learned using these models have translated into the human system and have provided keen insight for investigations using human subjects. We must rely on these models once again for controlled experiments that will address the mechanism by which cross-reactive T cells mediate disease pathologies. The knowledge gained by such research may eventually be used to circumvent, or shift the profile of, a cross-reactive T cell response before it causes disease or even to specifically target and destroy the

harmful cross-reactive T cells in the act. As a result, a new trend in medicine may emerge whereby each case is treated separately. Each individual differs not only by their genetic make-up but also by their immune system because of the unique number and sequence of exposures each person has to environmental pathogens.

CHAPTER 7:

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